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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **416th Meeting** *of the Society was held jointly with the Société Belge de Biochimie in the Physiological Institute, The University of Louvain, Louvain, Belgium, on Friday and Saturday,* 11–12 *May* 1962. *The Symposium was held on Friday,* 11 *May, and on Saturday,* 12 *May, the following Communications by Members of the Society were presented* (* *indicates that the Member received a grant from the Travel Fund of the Society*)*:*

SYMPOSIUM ON 'METHODS OF SEPARATION OF SUBCELLULAR STRUCTURAL COMPONENTS'

Introductory Remarks: the Scope and Limitations of Cell Fractionation

By C. DE DUVE. (*Laboratory of Physiological Chemistry, University of Louvain, Belgium*)

These introductory remarks will deal briefly with what may be called logical aspects of tissue fractionation, as opposed to the methodological aspects which form the main object of this symposium.

Destruction and reconstruction. The performance of a fractionation experiment involves three successive steps: from intact tissue or cells to homogenate; from the latter to isolated fractions; and from these to analytical results. These steps have to be retraced backwards when the data are interpreted.

The results of the measurements which have been carried out must first be related to the fractions, in the light of what is known of the analytical methods and of the possible artifacts involved. The composition of the fractions must then be evaluated in terms of the components of the homogenate, as defined exclusively by the physical properties which determine their behaviour during fractionation; the guiding principles here are the theoretical basis and practical limitations of the separation technique used. Finally, one can attempt to reinterpret the results in terms of specific intracellular entities, taking into account the known properties of the tissue and the consequences of homogenization and of exposure to a foreign medium.

In view of the numerous and distinctly different criteria which have to be applied at each stage, it is essential that this reconstruction process be accomplished in a truly stepwise fashion and without over-hasty extrapolation from the final results to the original material.

Preparative and analytical fractionation. Cell fractionation may be directed towards either a preparative or an analytical aim. In the former type, purity is the main objective and is generally approached at the expense of yield. Quantitative recovery is the essential prerequisite in the latter.

The application of preparative fractionation is limited by the sensitivity and accuracy of the methods whereby purity can be assessed. Morphological criteria are insufficient and must be complemented by biochemical criteria. This renders preparative fractionation largely dependent on prior analytical work of high enough resolution.

Analytical fractionation is most easily recognized as such when the fractionation scheme is arbitrary. In such a case, morphological evaluation of the fractions becomes almost impossible and cytological interpretation must be attempted on the sole basis of biochemical results. This can be done by making some assumption concerning the quantitative relationship existing between biochemical constituents and their host-particles. The preliminary conclusions reached in this manner can then serve to initiate further experiments, which will test their validity as well as that of the underlying assumption. Progress can thus be made, irrespectively of whether the assumption finally proves valid or not.

The classical fractionation schemes combine the analytical requirement for quantitative yield with the preparative strive after purity. They are pseudo-preparative analytical methods. They are particularly valuable, provided their essentially analytical nature is not obscured by preconceived ideas as to the purity and composition of the fractions. In particular, the possible presence in tissue homogenates of specific minor components, in addition to the major components on which the fractionation scheme is based, must not be overlooked.

Rationalization of methods. Current separation methods take advantage of differences in either sedimentation coefficient or density existing between different groups of particulate components. As will be emphasized by Beaufay in this symposium, the intrinsic physical properties of the particles which condition their separability, i.e. their size and density, and, accessorily, their shape, depend in a critical fashion on the composition of the medium and the relationship between

particle variables and medium variables is not necessarily the same for each group of particles. Consequently, particles which are not separable in one kind of medium may become so in another and further progress may be conditioned to a large extent by the search after new media. Even within the narrow range delimited by the stability of subcellular particles, enough variations are possible to warrant the statement that there probably exists an appropriate medium for each separation problem that may be encountered.

Nomenclature. With the simultaneous development of cell fractionation, electron microscopy and cytochemical staining techniques, a new language common to all fields of biology is being developed. It uses both old and new words, which are constantly gaining in precision and evolving in significance, as more criteria of identification are being established. The natural growth of this language is an important phenomenon which should be both helped along and kept under control. It is essential, in this respect, that the same word be prevented from acquiring different meanings in different areas. In particular, in the field of biochemistry, a clear distinction must be maintained between intracellular organelles or structures and subcellular fractions. To use the same term for both not only leads to semantic confusion, but may cause faulty interpretations and vitiate experimental designs.

Methods for Disrupting Cells

By D. E. Hughes† and V. R. Cunningham. (*Medical Research Council Cell Metabolism Research Unit, Oxford, and Medical Research Council Research Group on Demyelinating Diseases, King's College, Newcastle upon Tyne*)

In order to localize enzymic and other biological activity within cells by studying isolated organelles it is necessary to disrupt the cells in such a way that the organelles may be isolated and remain, in so far as is possible, both anatomically and functionally intact. Both physical and chemical methods are used to so disrupt cells and this communication will discuss mainly the physical methods with emphasis on the mechanism of disruption.

The precise mechanism of cell disruption by these physical methods is not known. This may be of little consequence in work with most animal tissues and protozoa where the forces employed are relatively small. With plant tissues and most micro-organisms, because of the strength of cell wall, the forces employed are very much greater and play an important role in determining the character of the disintegrated material obtained.

For instance, large molecules such as deoxyribonucleic acid are rapidly degraded by the high rates of shear used to disrupt bacteria by ultrasound but remain relatively intact when the cells are broken in solid extrusion presses. On the other hand, factors associated with oxidative phosphorylation appear to be missing in material from extrusion presses but present in some material prepared by ultrasound.

In the most commonly employed methods of cell disintegration the outer layers of cells are ruptured by liquid shear induced by turbulent flow. Sufficiently high shear rates to break up animal cells of many types may be generated by a variety of means. These include the rapidly revolving blades of Waring-type blenders, rapidly revolving cylindrical plunger or flow-past conical plungers. The shear rate is determined either by the speed of rotation and/or the clearance between plunger and vessel wall. A good deal of 'laboratory lore' exists for preparing cell organelles such as nuclei and mitochondria by these methods. Recently, more precisely controlled rates of shear can be generated by passing animal- and plant-cell suspensions through annular jets under pressure. Similarly, bacteria can be broken by flow at high pressures at a needle valve.

High rates of shear sufficient to disrupt bacteria are also generated when cell suspensions are rapidly shaken with small glass beads. The disintegrative effects depend on frequency and amplitude and are probably caused by streaming generated at the bead surface by water-hammer effects.

Cells of most types may also be broken by the passage of intense sound waves through suspensions or tissue aggregates. The disintegrative effects have been attributed to cavitation. This complicated phenomenon may be briefly defined as the development of gas and vapour-filled bubbles by the alternating pressures of a longitudinal sound wave. The bubbles exhibit complicated cycles of growth and collapse which generate, particularly at the collapse stage, high instantaneous temperatures (10000° K) and pressures (10^6 atm.) and electrical and chemical effects including free-radical formation. Evidence will be presented that cell rupture may occur in the absence of the deleterious effects associated with cavity collapse.

Methods employing solid shear such as is generated by grinding with abrasives are now used less for animal tissues than for plants and bacteria. Extrusion presses in which cells are mixed with abrasive or extruded frozen at $-25°$ are widely used for bacteria. The pressures used are very high (10^4 lb./sq.in.) and in the frozen cells disruption is

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caused partly by changes in ice-crystal volume and partly by shear. These methods are easily adapted to large scales. Disruption by explosive decompression, used mainly for breaking bacteria, could possibly also be used for other cells and tissues.

All of the methods described fall short of the ideal especially when working with micro-organisms. Some respects of this may be taken into account by comparing the results of several methods of disruption with the hope of obtaining complementary results.

A Comparative Account of Methods for the Isolation of Nuclei

By D. B. Roodyn. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Nuclei isolated by the 'perfect' method would (1) be undamaged morphologically, (2) be unaltered in chemical composition, (3) be obtained in excellent yield, (4) be free of contamination with non-nuclear material and (5) retain their full biological activity.

Morphology. Nuclei isolated by the various methods differ greatly in their volume, granularity, shape, sharpness of nuclear membrane and nucleoli, and extent of clumping. The appearance of the nucleus is profoundly affected by the ionic strength and pH of the medium and by the presence of divalent cations or basic substances (Anderson & Wilbur, 1952; Philpot & Stanier, 1956). Many (but not all) of the optical effects are freely reversible on changing the suspension medium (Schneider, 1955) and are probably derived from changes in the physicochemical properties of the contained DNA–histone complex. The extent of nuclear clumping in the different preparations is also related to these changes. Microscopic examination also reveals nuclear damage in the form of torn or pulled-out nuclear membranes, partially extruded nuclear contents, or even pieces of free nucleoplasm. Very little quantitative information is available as to the extent of such damage in the various methods. Electron microscopy has been used only in a few cases to demonstrate the retention of nuclear structure and the extent of damage inflicted, for example, to the complex pore structure of the nuclear membrane.

Chemical composition. Although information is available on the DNA, RNA, lipid and protein content of the various nuclear preparations, the significance of this is difficult to assess because of the confusing effects of possible cytoplasmic contamination and loss of soluble nuclear proteins during isolation. Because the nuclear membrane is probably freely permeable to substances of high molecular weight, the true soluble-protein content of the nucleus may be impossible to determine by bulk isolation methods and the value obtained after isolation purely a function of the strength of binding between soluble protein and DNA–histone under the particular conditions of pH and ionic strength used. There is probably insufficient evidence at the moment to be certain that the isolation of nuclei in organic solvents (Behrens, 1932) overcomes this difficulty.

Yield of nuclei. Yields can be determined by direct nuclear counts or by DNA determinations on the assumption that DNA is exclusive to the nucleus. In most methods employing aqueous media the reported yield is good, although sometimes yield is sacrificed in an attempt to reduce the contamination with whole cells. The information on nuclear yield in preparations with diluted citric acid (Dounce, 1943), or organic solvents, is limited, however.

Contamination. The various methods vary greatly in the extent of contamination with non-nuclear material and it is often difficult to obtain quantitative information of the amount present. Whole-cell contamination is possibly the most serious and variable form of contamination. In most methods the intact cells number less than 5 per 100 nuclei, but in some of the earlier methods using 0·25M- or 0·88M-sucrose the contamination was considerably more than this. Whole-cell contamination is greatly affected by the homogenization process and it is unfortunate that the physical forces applied to tissues during cell breakage are usually not accurately controlled or measurable. Mitochondrial contamination is readily measured either by direct count or by specific enzyme markers (e.g. cytochrome oxidase: Hogeboom, Schneider & Striebich, 1952) and is generally low. In certain methods, the media used disrupt the mitochondria so that possible contamination with mitochondrial fragments is difficult to assess. The use of specific enzyme markers for the measurement of contamination with lysosomal and microsomal material is limited, since it is not yet established whether the small amounts of these enzymes found in the nuclear fraction are due to nuclei or to contamination. Electron microscopy has been used in a few cases to assess contamination with endoplasmic reticulum but the results are difficult to express quantitatively. Contamination with adsorbed soluble cytoplasmic proteins is difficult to assess because of our ignorance of the true nuclear content of soluble protein. Red-blood-cell contamination in methods employing sucrose or glycerol media is readily estimated by direct count and is greatly reduced by perfusion of the tissue. With dilute

acid media or organic solvents the erythrocytes are lysed and no estimate is available of the possible contamination in these cases. Contamination with cell walls, fibre, and general 'cellular debris' can only be assessed qualitatively by microscopic examination, and there is insufficient information to compare the various methods.

Biological activity. Although the most satisfactory biological assay is to replace the isolated nucleus into an enucleated cell and to follow the recovery of the cell (cf. Burnstock & Philpot, 1956) this is unfortunately not possible with the tissues used for bulk isolation of nuclei. The ability to carry out certain complex biochemical processes (such as protein and RNA synthesis) has been demonstrated in certain isolated nuclear preparations (e.g. Allfrey, Mirsky & Osawa, 1957) with some evidence that these processes are destroyed by nuclear damage. At the moment, however, it is difficult to use such processes as quantitative indices of the retention of nuclear function. Of the various enzymes reported in the nuclear fraction, NAD pyrophosphorylase has the most convincing specific nuclear localization (Hogeboom & Schneider 1952). As it is released if the nuclei are damaged, it is a most useful quantitative index of nuclear function. Unfortunately it has only been measured in nuclei prepared by a few isolation procedures. Because of extreme difficulties in interpretation caused by the low amounts of enzyme frequently found in the nuclear fraction and the often unknown contribution of cytoplasmic contamination to the observed activity (Roodyn, 1959) the significance of the results on other nuclear enzymes cannot yet be assessed with certainty and these results certainly do not provide a clear picture of nuclear function.

A comprehensive appraisal of all the methods of nuclear isolation in the light of the above criteria is limited by lack of information, but it is clear that no one method satisfies all the requirements of a 'perfect' method listed in the beginning.

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The Influence of the Composition of the Suspending Medium on the Properties of Mitochondria

By J. B. Chappell* and G. D. Greville. (*Department of Biochemistry, University of Cambridge, and Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

Volume changes of isolated mitochondria are of three distinct but not necessarily unrelated types: (i) rapid adjustments to alterations in the osmotic pressure of the suspending medium; (ii) slow, large changes, occurring even in hypertonic media, induced by certain 'swelling agents'; and (iii) relatively small and rapid changes accompanying alterations in the redox state of the respiratory carriers.

(i) In these rapid water movements, mitochondria behave as ideal osmotic systems if they are reckoned to have an osmotic dead space equal to about one-half of their volume in isotonic sucrose (Tedeschi, 1961). Sucrose penetrates only partially into mitochondria, and it has been suggested that there is a limited space freely permeable to it. Malamed & Recknagel (1959) identify this with the osmotic dead space. On this view, mitochondrial water occupies two compartments; these would have to be identified in electron micrographs. In contrast, Tedeschi (1961) considers that the osmotic dead space is accounted for by the mitochondrial solids. Slow swelling in sucrose or salt solutions is believed to be an osmotic phenomenon consequent upon penetration of the external solute, such penetration necessarily involving entrance of water.

(ii) Rat-liver mitochondria in dilute suspension in 0·3 M-sucrose containing tris, pH 7·5, have been found by various workers to swell on incubation at room temperature, although in our experience they do not, at any rate in the first hour. It is widely recognized that addition of certain compound ('swelling agents') will induce or accelerate swelling; these include phosphate, thyroxine, Ca^{2+}, phlorizin, thiols, disulphides, oleate, ascorbate. Swelling induced by phosphate and thyroxine has been extensively studied and is now thought to be supported by electron transport. This is shown by the selective effects of respiratory inhibitors (e.g. swelling is inhibited by Amytal and then restored by succinate) and the need to add substrate in the case of mitochondria depleted of endogenous substrate by aging. Under certain conditions, however, substrate addition may delay swelling. It has been suggested that, because it can be prevented by 2,4-dinitrophenol, swelling depends on phosphorylation accompanying electron transport; this seems unlikely since under some circumstances dinitrophenol accelerates swelling, and it also

reverses inhibition of swelling by oligomycin, a potent inhibitor of oxidative phosphorylation. Swelling induced by Ca^{2+} or thyroxine, but not by phosphate or glutathione, is accompanied by formation of a fatty acid; this may be responsible for the swelling (see Hunter *et al.* 1959; Chappell & Greville, 1960; Wojtczak & Lehninger, 1961).

ATP can cause swollen mitochondria to contract, even under hypotonic conditions. Various additional cofactors are required, depending on the nature of the swelling agent used, e.g. phosphate-induced swelling is reversed by ATP together with Mg^{2+} or Mn^{2+} whereas glutathione-induced swelling requires the further addition of serum albumin and a contraction factor of mitochondrial origin. Contraction is inhibited by sucrose and other polyhydroxy compounds, azide and oligomycin, but not by 2,4-dinitrophenol. It is not dependent on electron transport, but its sensitivity to inhibitors of the partial reactions of oxidative phosphorylation indicates that the reversal process is connected with one stage of the oxidative synthesis of ATP, probably the terminal one (see Lehninger, 1959).

(iii) The addition of ADP to aerobic suspensions of heart and liver mitochondria in presence of phosphate and substrate leads to at least three changes: (*a*) rapid oxidation of pyridine nucleotides and cytochromes (other than *a*), (*b*) increase in respiration, and (*c*) shrinkage of the mitochondria. Mg^{2+} or Mn^{2+} is required, and Ca^{2+}, excess of phosphate or arsenate inhibit. When the added ADP has been converted into ATP the respiration and the state of reduction of the carriers return to their initial values and swelling begins. Added ATP does not have the same effect as ADP in this situation. Packer (1960) suggests that when the intramitochondrial concentration of ADP is high contraction occurs and when it is low, swelling.

The concept has arisen that intact mitochondria possess certain biochemical attributes, such as 'latency' of enzymes (e.g. ATPase, rhodanese, NAD-linked dehydrogenases) and respiratory control. In endeavours to produce mitochondria as little damaged as possible, as judged by these criteria, various workers have studied the effects of alterations in the constituents of the isolation medium. Efforts have also been made to correlate structural and biochemical integrity. Variations which have been tried include: (1) replacement of sucrose by raffinose or mannitol, (2) changes in sucrose concentration, (3) addition of macromolecules, and (4) addition of metal-chelating agents. While raising the sucrose concentration to 0·44M led to isolation from liver of rod-like mitochondria said to have well-preserved morphological features (cristae, double outer membranes, normal appearance of matrix), their biochemical properties were no better than those of the somewhat swollen, rounded mitochondria prepared with 0·25M-sucrose. Raising the sucrose concentration further to 0·88M led to rod-like particles with obviously abnormal appearance in the electron microscope and inferior biochemical properties. Rod-like mitochondria could be prepared even in 0·25M-sucrose if it contained substances of high molecular weight (dextran, polyvinylpyrrolidone, heparin), but again the biochemical properties were not improved. Some workers have included EDTA in the medium used for isolation of liver mitochondria, but apparently this is unnecessary with this tissue. With muscle, however, addition of a metal-chelating agent to the isolation medium is mandatory, seemingly to remove tissue Ca^{2+}. Beneficial effects of adding ATP or Mg^{2+} may be due to their property of binding or replacing Ca^{2+}, although possibly the ability of ATP to induce contraction of the mitochondria may be involved. An incidental advantage is that the myofibrils contract under these conditions and this allows a cleaner separation of the mitochondria. Inclusion of ATP, Mg^{2+} and EDTA permits preparation of mitochondria even in a saline medium (pigeon-breast muscle, embryo).

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The Separation of Subcellular Structures from Brain Tissue

By V. P. Whittaker. (*Biochemistry Department, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge*)

Electron-micrographic studies made recently by Dr E. G. Gray, and briefly reported by Gray & Whittaker (1962*a*), show that when slices of brain cortex are soaked in 0·32M-sucrose at 0° for 30 min. five characteristic structural changes take place. These are (1) a large increase in extracellular space, (2) swelling and disintegration of dendrites and cell bodies of neurones, (3) condensation of mitochondrial membranes with vesication and a 'zebra striped' appearance of the cristae, (4) spontaneous formation of microsomes in the cytoplasm, (5) the shrinking and pulling away of nerve endings from

their axon terminations and post-synaptic attachments. The response of the tissue is consistent with the dissolution of intercellular cement, perhaps by chelation of calcium ions by sucrose, the penetration of sucrose and water into cell bodies and dendrites and an ensuing transfer of water from the nerve endings to the cell bodies. Similar but less extensive effects are seen after 5 min. It would be interesting to know if other carbohydrates produce similar effects, especially inulin, which has been used to measure extracellular space. The changes induced by sucrose are in marked contrast with the effect of soaking in isotonic saline, which is simply to cause some swelling of the tissue without the separation and disintegration of cell membranes seen in sucrose.

Observations of the torn edges of tissue sections (Gray, 1959) show that the dendrites and surrounding glial processes are also mechanically less robust than nerve endings and tend to disintegrate under shear forces leaving the nerve endings intact. A combination of the effects of sucrose and mechanical shear could therefore account for the way in which nervous tissue breaks down when homogenized in sucrose. In addition to the release of nuclei and mitochondria, and the formation of microsomes and myelin fragments, nerve endings are torn or pinched off their attachment (Gray & Whittaker, 1960, 1962*b*) to form isolated nerve-ending particles (NEP's) about 1 μ in diameter with similar sedimentation characteristics in 0·32 M-sucrose to mitochondria. These particles appear as thin-walled bags packed with synaptic vesicles, the characteristic cytoplasmic organelles of the nerve ending about 500 Å in diameter; they also often contain one or more small mitochondria and may, in addition, have a short, thickened length of post-synaptic membrane still adhering to their periphery. The various particles formed by homogenization overlap in size, so that differential centrifugation alone cannot separate them. However, by combining this with density-gradient separation, nuclei, myelin, NEP's, mitochondria and microsomes can all be separated as distinct and relatively homogeneous fractions in good yield.

The isolation of NEP's opens up the possibility of a detailed study of the biochemistry of synaptic function, and recent progress along these lines will be described. By washing the particles repeatedly with distilled water, a membrane preparation can be obtained which is apparently free from cytoplasmic constituents and which is probably the purest preparation so far available of nerve membranes. Attempts have also been made to prepare isolated synaptic vesicles. Osmicated vesicles are released, together with fragments of osmicated cell membranes, if osmicated NEP's are subjected to gentle mechanical disintegration during dehydration (Whittaker, 1959; Gray & Whittaker, 1962*b*). In the native state, however, they remain clumped together as though embedded in a jelly-like cytoplasm, even when the outer membrane of the NEP is disrupted. It is thus doubtful whether the vesicles isolated by de Robertis, de Iraldi, Rodriguez & Gomez (1961) in the microsome fraction are in reality synaptic vesicles.

The isolation of NEP's demonstrates that subcellular fractionation techniques may be used to isolate not only intracellular organelles as such, but also functional portions of cells. Another illustration is the ingenious magnetic separation of renal glomerulae after iron embolism (Cook & Pickering, 1959). This approach to the study of cellular function will increasingly require close morphological control, usually at the electron-microscopic level, of the material being isolated. Unfortunately the conventional histological techniques for the electron microscope are extremely time-consuming; thus a biochemist may produce in one day enough material to keep an electron microscopist busy for weeks. We have therefore attempted (Horne & Whittaker, 1962) to adapt for the study of mammalian subcellular fractions the negative-contrast (negative-staining) method so successfully used for the study of viruses and bacterial cells (Horne, 1961). In this method, particulate material is simply mixed with 2 % phosphotungstic acid (PTA), previously neutralized to pH 7·4 and sprayed on to specimen grids with an atomizer which produces fine droplets of 1–10 μ in diameter. Under favourable conditions, the PTA forms an electron-dense glass in which the unstained particles show up as areas of low electron density. The particles are seen from above and not in section, and therefore may have an unfamiliar appearance; however, we have been able to identify all the main constituents of brain homogenates. Electron micrographs obtained by this method suggest that synaptic vesicles are distinct subcellular organelles, and resemble red cells in shape rather than ping-pong balls; they also confirm that disruptive treatments do not release the vesicles, and suggest that in some cases the effect of the disruptive treatment may simply be to cause the vesicles to fuse with the outer membrane. In work on membrane fractions the method has also proved extremely useful as a rapid check on the homogeneity of preparations after subfractionation. Some membrane structures not previously seen in homogenates have been revealed, notably large sheets of endoplasmic reticulum studded with ribosomes. The method may be used on fresh, formalin-fixed or osmicated particles; formalin-fixation is particularly useful for preserving mitochondria. These are often long, horseshoe-shaped and waisted as though in process

of budding off. The large amount of morphological detail obtainable by the new technique will undoubtedly complement that obtained by thin sectioning, and its inherently greater resolving power can be expected to furnish important new results.

The negative-contrast technique cannot as yet be applied to tissues. The survival in brain homogenates of discrete portions of cells provides material which enables it to be applied for the first time to organized cytoplasm.

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The Significance of Subcellular Fractionation as Applied to Certain Hydrolytic Enzymes

By J. Conchie and G. A. Levvy. (*Rowett Research Institute, Bucksburn, Aberdeen, Scotland*)

de Duve (for review, see de Duve, 1959) advanced the hypothesis that certain hydrolytic enzymes are confined together within a distinct type of cytoplasmic granule, the lysosome, which is somewhat lighter than the mitochondrion. The difficulties attendant upon the assignment of biochemical properties to specific microscopic entities at the subcellular level have not been overcome in the case of the lysosomes, and it is still possible that these are a heterogeneous group of particles overlapping the mitochondria and the microsomes. Information on the lysosomal particles is still dependent entirely on biochemical studies of centrifugal fractions from broken-cell preparations.

Hydrolytic enzymes that lack co-factors lend themselves to quantitative measurements of morphological features that influence their activity. In a study of β-glucuronidase in water homogenates of mouse liver (Kerr & Levvy, 1951; Walker & Levvy, 1951), about half the enzyme was bound to the cytoplasmic particles, in which form it was fully active. Bound enzyme could be released into the solution by incubation in acetate buffer at pH 5, by mechanical disintegration, by freezing and thawing, by treatment with detergents, or by prior treatment with acetone. It appeared that in a water homogenate the granules were surrounded by a protective fatty layer.

This study of β-glucuronidase was extended to homogenates of mouse and rat liver in isotonic media (Walker, 1952; Walker & Levvy, 1953). In such preparations only 5–15% of the enzyme was now found in the extra-particulate fraction. The remainder was associated with cytoplasmic granules of all sizes, from which it could be released by the measures already described. Making the medium hypotonic caused a progressive release of enzyme to reach ultimately the percentage seen in water homogenates.

Preparations in isotonic media displayed only a fraction of their total activity, i.e. they displayed 'latency'. Release of the enzyme from the granules was accompanied by activation of the preparations. It was concluded that in isotonic media—as opposed to hypotonic media—the membrane or interface surrounding the granules prevented free access of substrate. This barrier was partly overcome by increasing the substrate concentration.

The behaviour of β-glucuronidase in isotonic preparations of liver bore a remarkable resemblance to the results that de Duve and his colleagues had obtained for acid phosphatase in rat liver (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951). There were, however, differences between β-glucuronidase and acid phosphatase, which influenced the general interpretation of the results.

β-Glycerophosphate, the substrate used for acid phosphatase, was the only compound examined that could replace sucrose in the artificial medium and maintain the enzymic integrity of the granules. No increase in their acid-phosphatase activity was observed on increasing the substrate concentration (Appelmans & de Duve, 1955). Apart from the small fraction of the enzyme found in solution, the acid-phosphatase activity displayed by preparations in isotonic media was considered to be entirely attributable to adsorbed soluble enzyme. There was, however, evidence for slow penetration of the granules by β-glycerophosphate. Acid phosphatase appeared to be associated with particles with a sharply-defined mean sedimentation rate, rather lower than the mean value for mitochondria (Appelmans, Wattiaux & de Duve, 1955).

Gianetto & de Duve (1955) found that β-glucuronidase, acid phosphatase and cathepsin were activated at the same rate in the rat-liver mitochondrial fraction, and it was concluded that

the enzymes were enclosed together within the same particle. This work was extended to other hydrolytic enzymes, and the term lysosome was proposed for the particle concerned. The other enzymes, however, did not always sediment at exactly the same rate as acid phosphatase: for example, considerable β-glucuronidase activity was found in the microsomal fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

According to some interpretations of the lysosome theory, the enzymes concerned are confined behind impervious barriers throughout the body, and only become active on the death of the cell. The most important criticism of this 'lethal-bag' theory is that it generalizes from results obtained almost exclusively with liver.

The study of the subcellular localization of β-glucuronidase in isotonic homogenates has been extended to other tissues (Conchie, Hay & Levvy, 1961). A homogenizing technique had to be developed to compare tissues of varying intractability. Mouse kidney resembled liver in that the β-glucuronidase activity was associated with the mitochondrial and microsomal fractions. With mouse spleen and cancer tissue, on the other hand, about half of the enzyme appeared in the extra-particulate fraction.

Whereas, in the case of mouse tumours, the activity of the whole sucrose homogenate was largely accounted for by the soluble enzyme, the overall activity in the case of mouse spleen was too high to be completely accounted for in this way. Very high percentages of the total activity were also seen in isotonic homogenates of C3H-mouse liver and pig liver, although the figures for soluble enzyme in these tissues were little greater than in ordinary mouse liver. Pigs and the C3H strain of mice are notable for their very low β-glucuronidase activity throughout the body. It may be that this is compensated for by increased permeability of the granules towards substrate, as measured in isotonic homogenates.

The distribution of β-galactosidase, α-mannosidase and β-*N*-acetylglucosaminidase has also been studied in sucrose homogenates (Conchie & Levvy, 1960; J. Conchie & A. J. Hay, unpublished work). These enzymes, which also displayed latency, resembled β-glucuronidase in their distribution in mouse liver and kidney. In mouse spleen and cancer tissue, however, β-*N*-acetylglucosaminidase was largely confined to the microsomal fraction; the other two enzymes in these two tissues were distributed between the microsomal and soluble fractions.

Rat liver and rat spleen closely resembled the corresponding mouse tissues in the distribution of β-glucuronidase, β-galactosidase, α-mannosidase and β-*N*-acetylglucosaminidase, except in one particular. In rat spleen, α-mannosidase was largely confined to the microsomal fraction.

Although it is not yet possible to decide whether the lysosomes form a separate class of cytoplasmic particle, they are evidently heterogeneous as to function and size. An enzyme that has been found to be particulate in one tissue may be free in the extra-particulate fraction in another. Even with a particulate enzyme, latency need not represent an absolute barrier to activity *in vivo*, since its magnitude can vary from tissue to tissue.

Useful preliminary tests for work in this field are: (*a*) separation of the enzyme in a sucrose homogenate into particulate and soluble fractions by buffering with acetate to pH 5 and centrifuging at ordinary speeds, followed by measurements of the total activity in both fractions in the presence of Triton X-100; (*b*) measurement of latency in the whole sucrose homogenate by measuring its activity in the absence and presence of Triton X-100.

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Techniques for the Isolation of Particulate Glycogen and its Examination in the Electron Microscope

By P. Drochmans. (*Laboratoire de Cytologie et de Cancerologie Experimentale, Université Libre de Bruxelles*)

The classical method of differential centrifugation used for the isolation of particulate glycogen has been modified to simplify and accelerate the procedure and to obtain a purer undegraded compound. The particulate glycogen obtained by means of this method has been examined with

electron-microscopic techniques giving a high contrast.

The method of preparation consists in lowering the pH of a liver extract down to 5·2. At this pH value, mitochondria and microsomes agglutinate and the aggregated particles sediment readily at low-speed centrifugation (1100*g* for 5 min.). The glycogen is unaffected by this slight acidification and remains dispersed in the supernatant. This particulate glycogen may be separated from the supernatant by high-speed centrifugation (50 min. at 20 000*g*) as a compact, transparent pellet. At this stage of separation, the glycogen is still contaminated by enzymes capable of degrading it, as shown by incubation tests at 37°. This effect is shown by a proportional decrease in the opalescence of the glycogen suspension. This breakdown, which is accelerated at specific pH's, may be progressively decreased by repeated washing of the glycogen preparation in dilute buffer solutions at pH 9·0. Elementary analysis has shown that the glycogen prepared by the method indicated has lower nitrogen and phosphorus content (0·01 % and less than 0·01 %, respectively) and is appreciably purer than that obtained by the classical chemical techniques.

Electron-microscope studies of the particulate glycogen and its breakdown products reveal that examination of the morphology of the glycogen and of other polysaccharide preparations may be a powerful tool in testing their purity and homogeneity. Two techniques have been applied to the study of the morphology of the glycogen particle: (1) positive staining which consists of treating the glycogen suspended in agar with potassium permanganate; ultra-thin sections in the agar–methacrylate-embedded material show under the electron microscope the fine structure of the glycogen particles; (2) negative staining which consists in examining a thin film of glycogen suspended in potassium phosphotungstate. In this latter procedure, glycogen in its original particulate state or in degraded forms appears relatively transparent to electrons, in contrast with the surrounding, strongly electron-scattering phosphotungstate. These two complementary techniques have shown that the macromolecular organization of the glycogen particle corresponds to a three-stage structure. Particles exposed to acids below pH 5·0 undergo a stepwise dissociation which takes place at definite pH levels, leading to the formation of smaller glycogen units of definite and smaller sizes of the order of 150, 30 and 3 mμ in width, respectively.

Further studies, using the electron microscope techniques just mentioned, have been applied successfully to the analysis of the mechanism of polysaccharide synthesis from the morphological standpoint.

The Separation of Ribosomes and Oxidosomes from Bacteria

By J. De Ley. (*Laboratory for Microbiology, Faculty of Sciences, State University, Ghent, Belgium*)

Cytoplasmic membranes of strictly aerobic bacteria contain many enzymes (see reviews by De Ley, 1961*a*, *b*). This calls for extensive physical and chemical studies of this cellular substructure, which is largely unknown. By fragmentation a variety of particles are obtained, those bearing oxidative enzymes have been called 'oxidosomes' (De Ley, 1960). Disruption of Gram-negative cells or 'protoplasts' also releases ribosomes. Both types of particles are sedimented simultaneously by ultracentrifugation and are not easily separated.

Several physical methods were investigated as a tool for separating both types of particles in order to obtain pure preparations of each, for the preparation of large amounts and as an analytical tool to distinguish both.

Acetic acid bacteria were used, particularly *Gluconobacter liquefaciens*. 'Protoplasts' were prepared by the human-serum method and disrupted by short ultrasonic treatment. 'Hull' (wall plus membrane) fragments were prepared by ultrasonic or mechanical disruption of intact cells. Particles were sedimented by centrifuging at 100,000 *g* for 300 min. Tris–Mg^{2+}–succinate (TMS) buffer, pH 7·1, was used throughout.

(1) *Density-gradient centrifugation.* Very neat separation was obtained in TMS–0–70 % sucrose linear gradient. After 2–3 hr. at 25 000 r.p.m. in the rotor SW-25 three protein layers were obtained. The original upper layer contained minor traces of soluble protein. The middle one, which had reached the level of about 15 % sucrose, contained the ribosomes but also some very small oxidosomes. The lower layer stabilized at a density of about 1·24 and contained the oxidosomes or the 'hull' fragments, according to the experiment. Similar separations were also obtained in 0–35 % lactose gradients, where the oxidosomes were deposited on the bottom of the tube. Use of NaBr or CsCl gradients resulted in the same picture; however, the oxidosome fraction was now entirely devoid of RNA (see below).

(2) *Density-layer centrifugation.* Convenient separations can be carried out by suitable combination of uniform density layers, *g* and centrifugation time. By centrifugation of a layer containing both particles on top of a 20 % sucrose column, with a short column of 70 % sucrose in the bottom, the oxidosomes go as far as the 70 % column; the ribosomes penetrate to the top of the 20 % column.

(3) *Density-gradient electrophoresis.* An LKB column apparatus with various sucrose gradients

and electric fields was used. Electrophoresis for 20 hr. at 4°, 500 v and 12 ma in a 0–20 % linear sucrose gradient in TMS buffer, pH 7·5, provided a neat separation, with the ribosomes moving fastest.

(4) *Phase distribution.* The method according to Albertsson(1960) was investigated. Best separation was obtained in a biphasic system containing sodium dextran sulphate and methylcellulose. The ribosomes accumulate in the top phase; oxidosomes are concentrated in a small bottom phase.

The comparison of the above methods points to density-layer centrifugation as the most convenient method on the laboratory scale. Phase distribution is useful for mass production and can be followed by further purification on layers or gradients. Density-gradient centrifugation is more convenient than density-gradient electrophoresis as an analytical method.

The purified oxidosome fraction is now under further investigation. It still contains about 7 % of RNA which is tightly bound to it and which is always present, no matter how the oxidosomes are purified. The RNA can be solubilized by EDTA treatment. By prolonged ultrasonication RNA-containing particles are dislodged, representing either ribosomes, which are tightly bound to the cytoplasmic membrane, or RNA sites which are part of the membrane. It is not excluded that they are the sites where the biosynthesis of the membrane occurs.

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Medium Composition and Equilibrium Density of Subcellular Particles from Rat Liver

By H. Beaufay. (*Laboratory of Physiological Chemistry, University of Louvain, Belgium*)

Density equilibration in a density gradient (isopycnic centrifuging) has become a useful and often indispensable complement of tissue fractionation by differential centrifuging. It allows a more detailed analysis of the composition of the fractions and a more accurate localization of enzymes, may in many cases lead to a considerable purification of organelles of rare occurrence, and provides additional information concerning the physicochemical properties of subcellular particles.

The density of subcellular particles is known to be dependent on the composition of the suspending medium (de Duve, Berthet & Beaufay, 1959). The manner in which particles react to changes in medium composition varies from one type to the other, according to the permeability and elasticity of their limiting membrane, their content in osmotically active substances and the specific gravity and degree of hydration of their chemical constituents.

Separation of two groups of particles may often be achieved by taking advantage of these differences and may require a preliminary systematic search after appropriate conditions. In order to illustrate the influence of medium composition on equilibrium density, it will be shown how the various particulate populations present in rat-liver mitochondrial fractions are distributed in a number of density gradients. These studies have revealed the presence in such fractions of at least three distinct groups of organelles: mitochondria, lysosomes and other structures bearing urate oxidase, catalase and D-amino acid oxidase (Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959; de Duve *et al.* 1960).

Sucrose gradients. In such gradients, particles are exposed to media of extremely high as well as variable osmotic pressure; in spite of these drawbacks, they have frequently been used successfully. In aqueous-sucrose gradients, the lysosomal enzymes are distributed around a density of 1·22, while catalase, urate oxidase and D-amino acid oxidase tend to be grouped in a region of somewhat higher density, ranging between 1·23 and 1·25. The equilibrium position of the mitochondria depends on their initial position. If they have been layered in isotonic sucrose on top of the gradient, they tend to band in a narrow zone of density somewhat lower than 1·19; if they have been uniformly distributed within the gradient, those which have been exposed to sucrose concentrations higher than about 1·4 M (density 1·19) become concentrated around a density of 1·22 and their separation from the other particles is mediocre.

Replacement of the solvent water by deuterium oxide increases the equilibrium density of all particles, but nevertheless decreases the isopycnic sucrose concentrations. The average density values are 1·25 for the lysosomal enzymes and 1·26 to 1·27 for catalase, D-amino acid oxidase and urate oxidase. According to their initial position, the mitochondria migrate towards density regions of 1·23 or 1·25; in most cases, few particles occupy the latter position, since a 1·4 M concentration now corresponds to a density of 1·26. The separations achieved are generally good, especially when the material has been layered on top of the gradient.

Gradients of macromolecular substances. It is often advantageous to isolate subcellular organelles in isotonic media and to avoid exposing them to changes of osmotic pressure. Unfortunately, few macromolecular substances are suitable for the

preparation of density gradients. We have mostly used glycogen, with aqueous sucrose as solvent. Several sucrose concentrations have been tried: 1·273, 1·00, 0·561 and 0·264 molal.

As the sucrose concentration is lowered, the equilibrium density of the particles decreases according to a relationship which is different for each particle type. However, none shows a behaviour strictly compatible with a simple osmotic model limited by a membrane impermeable to sucrose. Lysosomes approach such a model most closely; on the other hand, the particles containing catalase, D-amino acid oxidase and urate oxidase seem to be largely accessible to sucrose. Thanks to this property, the latter particles become the least dense constituent in 0·561 molal sucrose and dissociate clearly from lysosomes in this medium.

In a few experiments, we have used 'Ficoll', a polymer with a molecular weight of approximately 50 000 introduced by Holter & Møller (1958) and manufactured by Pharmacia A.B., Uppsala, Sweden. In 0·561 molal sucrose, Ficoll gradients are of little practical value, since, for reasons as yet unexplained, all the particles are much denser in the presence of high concentrations of this substance than they are in the presence of glycogen, and become concentrated together at the bottom of the tube.

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COMMUNICATIONS

Gel Filtration of Proteins, Nucleic Acids and Polysaccharides

By P. ANDREWS* and G. P. ROBERTS. (*National Institute for Research in Dairying, Shinfield, Reading, Berks.*)

Gel filtration, developed largely by Porath, Flodin and their co-workers, is a fractionation method which could in principle be applied to molecules of all shapes and sizes. The commercially available gel-filtration media effect little separation of molecules with molecular weights above 40 000, so more open gels are needed to extend the technique to larger molecules. Polson (1961) has used columns of granulated agar gel to separate proteins of molecular weights up to 6 600 000. Since these gels are easily prepared, they are useful for investigating the gel filtration of large molecules.

The separation of proteins ranging in size from lactalbumin (mol.wt. 17 400) to thyroglobulin (mol.wt. 630 000) was attempted with gels containing from 2·5% to 12% by weight of agar. The best separations of protein molecules in different size ranges were obtained with gels of different concentrations, the more dilute gels separating the larger molecules, so that in applying the technique to specific problems a selection of gel concentration according to the size of solute molecules is advisable. The results of Polson (1961) are insufficient to make this point clear.

When goat-liver nucleic acid, prepared by phenol extraction of the homogenized tissue, was fractionated on a 5% agar column, the elution diagram showed three peaks, one of which coincided in position with purified yeast s-RNA (mol.wt. approx. 30 000), whereas the other two represented much larger molecules (Rosenbaum & Brown, 1961). A peak corresponding to s-RNA was also found on the elution diagrams of crude particle-free tissue extracts in other experiments.

The polydispersity of polysaccharides resulted in their giving wider bands than did proteins during filtration through agar columns. However, when examined separately, starch, clover-seed galactomannan, larch galactan and *Strychnos* galactan were eluted in different positions relative to protein standards, in the order listed. Bovine cervical mucoid and some of its degradation products, which were to a large extent irreversibly adsorbed on chromatographic media based on dextrans or cellulose, were separated and recovered quantitatively after passage through agar columns.

Gel filtration may provide a simple but valuable method for estimating approximate molecular weights and polydispersity of polysaccharides. Once the gel-filtration properties of polysaccharides for which these are known have been compared with those of suitable readily-available proteins, the latter can be used as reference standards for comparison with other polysaccharides.

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Oxidation of [1-^{14}C]Acetate and D-[U-^{14}C]-Glucose by the Testes and Epididymis of the Ram *in vivo*

By E. F. ANNISON, T. W. SCOTT*† and G. M. H. WAITES. (*Faculty of Rural Science, University of New England, Department of Veterinary Physiology, Sydney and C.S.I.R.O., Prospect, New South Wales, Australia*)

Isotope-dilution techniques provide the most accurate method of measuring substrate utilization in the intact animal and have been used extensively to study acetate and glucose turnover in many species (Baker, Shipley, Incefy & Miller, 1954; Searle, Strisower & Chaikoff, 1954; Steele, Wall, de Bodo & Altszuler, 1956; Annison & Lindsay, 1961; Annison & White, 1961). This technique has been used in the present studies to assess quantitatively the relative importance of acetate and glucose in the overall oxidative metabolism of the testes and epididymis of the anaesthetized ram.

[1-^{14}C]Acetate and D-[U-^{14}C]glucose were continuously infused through polythene catheters inserted in the right external jugular vein. Blood samples were withdrawn through similar catheters placed in the femoral artery, the left jugular vein, the left and right internal spermatic veins.

Arteriovenous differences in the specific radioactivities of blood carbon dioxide were related to the carbon dioxide produced by the gland and the data used to calculate the percentage contribution of acetate and glucose to the oxidative metabolism of the epididymis and testes. These calculations were based on the assumption that equilibrium conditions (i.e. a constant specific activity of plasma acetate or glucose) prevailed in the whole animal before sampling commenced (cf. Annison & Lindsay, 1961; Annison & White, 1961).

Comparison of the specific activities of carbon dioxide in arterial and venous blood during the infusion of $NaH^{14}CO_3$ indicated the occurrence of bicarbonate pools in both testis and epididymis. Constant relationships between venous and arterial carbon dioxide specific activities were reached after infusion for about 200 min. Possible substrate pools in the glands were investigated by comparison of the specific activities in arterial and venous blood shortly after the start of isotopic substrate infusions.

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The Incorporation of the Radioactivity of [3-^{14}C]Serine into Brain Glycerophospholipids

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It has been known for some time that L-serine can provide carbon atoms 1 and 2 of ethanolamine and choline (Arnstein, 1951; Elwyn, Weissbach, Henry & Sprinson, 1955). More recent work has suggested that, in the liver, decarboxylation and subsequent *N*-methylation can take place whilst the carbon chain is still attached to phosphate in the form of a lipid (e.g. Bremer, Figard & Greenberg, 1960). The decarboxylation of phosphatidylserine *in vitro* has recently been demonstrated by Borkenhagen, Kennedy & Fielding (1961) with liver homogenates.

To study the incorporation of the label of L-[3-^{14}C]serine into brain glycerophospholipids *in vivo*, rats (3–4 weeks old) were injected intraperitoneally with 0·2 μc/g. body weight. After exchange periods of 1–24 hr. the glycerylphosphoryl esters, obtained after mild saponification of the brain phospholipids, were separated by the method of Dawson (1954). Total radioactivity in the esters, and its distribution in the glycerophosphate and base after acid hydrolysis, were determined by scintillation counting.

After 2 hr. more than 20 % of the radioactivity in total brain lipids was found in the phospholipid serine and the specific radioactivity of this serine was 30–40 % of that of the free serine in the brain; this could indicate a rapid incorporation or exchange in the young rat. The specific radioactivities of the lipid ethanolamine and choline were respectively 6 % and < 3 % of lipid serine. The ratio of the specific radioactivity of the base to that of the glycerol moiety after 2 hr. was 8·5 for phosphatidylserine, approx. 2 for phosphatidylethanolamine and $< 0{\cdot}05$ for phosphatidylcholine. After 4 hr. the ratios were 9·6, 5·4 and 0·24 respectively.

These results suggest that the glycerol of brain phosphatidylcholine does not derive from phosphatidylserine since, if this were so, these ratios would be very similar. Further support for this came from experiments in which 0·4 μc of DL-[3-^{14}C]serine/g. was injected; this yielded more

radioactivity in the glycerol of phosphatidylcholine than 0·2 μc of L-[3-^{14}C]serine/g. indicating that D-serine can supplement the formation of phospholipid glycerol (cf. Minthorn, Mourkides & Koeppe, 1959). That the pathway of Bremer *et al.* (1960) is not important in the brain *in vivo* might also be inferred from the earlier observation of Ansell & Dohmen (1957) that the specific radioactivity of phosphatidylserine, a short time after [^{32}P]orthophosphate injection, was the lowest of all the glycerophospholipids.

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The Stimulation of Oxygen Uptake of Myxoedematous Leucocytes *in vitro* by Tri-iodothyroacetic Acid

By SHEENAH K. BISSET and I. P. C. MURRAY. (*University Department of Medicine, Royal Infirmary, Glasgow, C.* 4)

The oxygen uptake of leucocytes of myxoedematous patients is influenced by the level of the thyroid hormones circulating in the blood stream (Alexander & Bisset, 1961). It has been shown that both tri-iodothyronine (T_3) and tri-iodothyroacetic acid (TRIAC), injected intravenously in equivalent amounts into a myxoedematous patient, will have a stimulatory effect on the oxygen uptake of leucocytes but that TRIAC will produce a more rapid and greater stimulation (Bisset & Alexander, 1961).

However, in studies *in vitro* of the oxygen uptake of myxoedematous leucocytes with the direct method of Warburg with a modified Krebs no. 2 buffer, an immediate stimulation can be demonstrated with TRIAC but no effect is produced by T_3.

The explanation for these conflicting results may be: (*a*) In studies *in vitro* the leucocytes were not washed. Since T_3 has a greater binding capacity for protein than TRIAC (Tata, 1960), the added T_3 might be bound to serum proteins with a consequent reduction in the amount of free hormone available for metabolic action. (*b*) T_3 may be converted *in vivo* into TRIAC which, as the active metabolic agent, then stimulates the oxygen uptake.

Both these possibilities have been investigated. The first appears unlikely, since thorough washing of the leucocytes to remove contaminant serum protein did not induce any stimulation of respiration by T_3.

When, however, myxoedematous leucocytes were incubated *in vitro* with T_3 together with L-amino acid oxidase, the oxygen consumption was increased over the control to a degree comparable to the stimulation obtained with TRIAC. This apparent conversion of T_3 by deamination to TRIAC or to a compound with a metabolic activity similar to that of TRIAC provides further evidence of the importance of this pathway in the degradation of the thyroid hormones.

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The Reversible Activation of Lysosomes in Normal Cells

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Lysosomes are inert particles when isolated under ideal conditions in tissue fractions, since the acid lytic enzymes are contained within a lipid–protein membrane, and are released only when this membrane is damaged (for example de Duve, 1959). Although Holt (1959) demonstrated lysosome-like structures in rat-liver and kidney sections, his techniques did not enable him to show that these were inert particles which became activated only when the membrane was modified.

Particles which fulfil all the criteria of the biochemists' lysosomes have been demonstrated in sections prepared by the controlled temperature freeze-sectioning procedure (Cunningham, Bitensky, Chayen & Silcox, 1959). The activity of acid phosphatase, the marker enzyme, was demonstrated histochemically by the Gomori procedure, suitably modified for cryostat sections. The particles were inert, but they could be activated by incubation at 37° at pH 5, by procedures similar to those used by de Duve and by treating the sections with formalin before incubation. The incubation time necessary to render the normal lysosomal membrane permeable to glycerophosphate has been measured and damage *in vivo* to this membrane was detected

because of its altered permeability and increased fragility.

Thus rat-liver lysosomes were found to be activated within a few hours of the administration of an ether anaesthetic. Greater activation was caused by a simple laparotomy or by handling the liver. These membrane modifications did not allow the escape of the hydrolytic enzymes *in vivo*, since no necrosis was observed either concurrently with or following these lysosomal effects; moreover these changes underwent regression. In addition, reversible lysosomal activation was demonstrated in smears of mouse ascites-tumour cells subjected to active hetero- or iso-antibodies in the absence of complement. The alteration of the lipid–protein membranes was shown by the unmasking of phospholipid in cell particles which appeared identical to the lysosomes.

Lysosomes have been considered to be 'suicide particles' which when activated cause the death of cells. It will be argued that the reversible activation, without release of enzymes *in vivo*, found in this study might reflect a more advanced stage of normal permeability changes of the lysosomal membranes.

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The Effect of Long-Chain Fatty Acids on Glucose Utilization in the Isolated Perfused Rat Heart

By R. H. Bowman.† (*Department of Biochemistry, University of Cambridge*)

In hypophysectomized rats, cardiac glycogen is increased when oleic acid is fed (Bowman, 1959). In rat hearts perfused *in vitro*, Newsholme, Randle & Manchester (1962) found that ketone bodies and octanoate increase glycogen, impair the phosphorylation of glucose and fructose 6-monophosphate, and decrease glucose uptake. The present report concerns the action of long-chain fatty acids on uptake and intracellular accumulation of glucose in the perfused rat heart.

Bovine serum albumin (Armour) was employed as a carrier for the fatty acids. To avoid introduction of long-chain fatty acids into control perfusates, the albumin was previously freed of endogenous fatty acid by a modification of the heptane–acetate extraction procedure of Goodman (1957). After extraction the albumin was dialysed exhaustively and freeze-dried. Perfusates were constituted by preparing a 3% albumin solution in Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932). Fatty acids, as the potassium salts, were added to the albumin medium which was then dialysed against a large excess of bicarbonate buffer for 36 hr. before use. Immediately before perfusion, glucose (1 mg./ml.) and insulin (0·1 unit/ml.) were added to the perfusates.

Rat hearts were perfused (Morgan, Henderson, Regen & Park, 1961) for 15 min. by recirculation with 15 ml. of albumin solution ± fatty acids. In the presence of insulin it was found that palmitate, oleate and linoleate at concentrations of 0·3 to 2·0 mM increased intracellular free glucose and decreased glucose uptake (intracellular glucose space increased from control levels of 8 ml./100 g. to approximately 20 ml./100 g., and glucose uptakes decreased 20 to 30% from control values of approximately 10 mg./g./hr.). It is concluded that long-chain fatty acids decrease the rate of glucose phosphorylation in the rat heart. In the absence of insulin these effects were not observed. This may be due to low glucose uptakes in non-insulinized hearts, which tends to make phosphorylation not rate-limiting (Morgan *et al.* 1961). However, this is contrary to a report of Shipp, Opie & Challoner (1961) noting an inhibition of glucose uptake by palmitate in the rat heart perfused without insulin.

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The Extraction and Separation of Isoenzymes of Aspartate Transaminase

By J. W. Boyd.* (*Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

Fleisher (1960) purified isoenzymes of L-aspartate–α-oxoglutarate aminotransferase (EC 2.6.1.1)

from dog liver by chromatography, heat treatment and $(NH_4)_2SO_4$ fractionation. Subsequently it was found that isoenzyme I was localized in the supernatant fraction of rat-liver homogenates, while isoenzyme II was tightly bound in the mitochondrial fraction (Boyd, 1961*a*, *b*). The extraction of these isoenzymes from other rat tissues has been studied.

The extraction of bound isoenzyme II achieved by blender treatment was 43, 29, 12 and 5% from liver, kidney, heart and skeletal muscle respectively. Butan-1-ol has been found to be much more effective, extracting over 90% of the bound isoenzyme from each of these tissues. Since many other proteins are selectively denatured by butan-1-ol, this treatment effects considerable purification. Further purification has been achieved by $(NH_4)_2SO_4$ fractionation. Two overlapping peaks of activity have been found in $(NH_4)_2SO_4$ fractions from butan-1-ol extracts of rat-liver homogenates. One peak occurred in the 60–75% and the other in the 65–90% saturated fractions. There was some enzyme still in solution at 100% saturation. The first peak consisted mainly of isoenezyme I and the second of isoenzyme II. Butan-1-ol-treated rat-liver supernatant, consisting mainly of isoenzyme I, gave only the first peak on fractionation. A butan-1-ol extract of mitochondria, consisting mainly of isoenzyme II, gave the second peak and some unprecipitated activity.

The relative proportions of the two isoenzymes during purification was determined using the differential-assay method (Fleisher, Potter, Wakim, Pankow & Osborne, 1960) and photoautographs of isoenzymes on agar gels after electrophoresis (Boyd, 1961*c*, 1962). The substrate layer, for the latter method, contained 10 mM-α-oxoglutarate, 80 mM-L-aspartate, 1 mM-NaDH, 5 μg. of malate dehydrogenase/ml., 0·4% agar and 80 mM-phosphate buffer, pH 7·5. Scanning the photoautographs in a recording densitometer gives an estimate of the relative proportions of the isoenzymes. The densitometer can be modified for use as a fluorimeter to scan the gels themselves. An ultraviolet filter is placed in the light beam and the fluorescent light, emitted by the NADH in the gel, is recorded during the scanning. This method could be used for locating isoenzymes of any enzyme which involves, or can be coupled with, oxidation or formation of fluorescent compounds.

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Control of Porphyrin Biosynthesis through a Negative-Feedback System

By B. F. Burnham. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The photosynthetic bacterium *Rhodopseudomonas spheroides*, when cultured in the light under iron-deficient conditions, accumulates large quantities of porphyrin in the medium; addition of iron results in a decreased production of porphyrin (Lascelles, 1956, 1960). The effect of iron seems to be catalytic and not the result of the formation of a stoicheiometric amount of haemin. Haemin has been observed to repress the formation of two enzymes required for porphyrin synthesis, δ-aminolaevulic acid (ALA) synthetase and ALA dehydrase (Lascelles, 1960). Haemin was also found to inhibit the activity of ALA synthetase in crude sonic extracts (Gibson, Mathew, Neuberger & Tait, 1961).

The present investigation was undertaken to examine the possibility that iron added to deficient cultures exerted its apparent catalytic influence upon porphyrin formation after being incorporated into haemin, which, in turn, inhibited the activity of an enzyme concerned in porphyrin synthesis through a negative-feedback mechanism. In other negative-feedback systems, the final product in a metabolic sequence inhibits the first enzymic step leading directly and exclusively to the end product.

The present results indicate that such a negative-feedback system may operate as one of the mechanisms for control of porphyrin synthesis.

ALA synthetase having a specific activity of 2 μmoles of ALA/hr./mg. at 37° has been prepared from *R. spheroides*. This represents about a 20-fold purification, and this material was used in all inhibition studies.

Protohaemin IX was a relatively potent inhibitor of ALA synthetase, causing about 50% inhibition at a concentration of 10^{-5}M. The specificity of the inhibition was examined by measuring the activity of the enzyme in the presence of several porphyrins and their metal complexes. The inhibition is quite specific for protohaemin IX. Iron haematoporphyrin, cobalt protoporphyrin, manganese protoporphyrin, and magnesium protoporphyrin had very little inhibitory action. The corresponding free porphyrins caused only slight inhibition. The inhibition caused by protohaemin IX was reversible.

The results of this preliminary investigation indicate that the inhibition of ALA synthetase by haemin may be of physiological importance in the control of porphyrin synthesis. The fact that this inhibition is apparent at very low concentrations of haemin, that the inhibition is reversible, and that it is specific, all support this possibility.

This work was aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

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The Reduction of 9α-Chloro-11-Oxo Steroids by Preparations of Rat Liver

By I. E. Bush and Sheila Hunter. (*Department of Physiology, The Medical School, Birmingham* 15)

It has been shown previously that the introduction of a 9α-fluorine atom into several 11-oxo steroids results in a great increase in the rate at which such steroids are reduced *in vitro* to the related 11β-ols by preparations of rat liver. Similarly, this substitution prevents almost completely the oxidation of the 11β-ols by such preparations under oxidizing conditions (Bush, Meigs & Hunter, 1962). The same effect is produced by introducing a 12α-fluorine atom, but a 12α-bromine atom prevents completely the reduction of an 11-keto group. The complete blocking of the reaction by the 12α-bromine atom and the fact that the increase in the rate of reduction of 11-keto groups due to the introduction of a 12α-fluorine atom is only about half that due to a 9α-fluorine atom confirm the theory of Bush & Mahesh (1959) concerning the stereochemical nature of the enzyme–substrate complex of this reaction. According to this theory, the introduction of a 9α-chlorine atom should cause little or no steric hindrance to the formation of the enzyme–substrate complex and the negative inductive effect of the chlorine atom should cause a moderate increase in the rate of the reductive reaction. We have therefore examined the reduction of 9α-chloro-11-oxo steroids.

Microsomes were prepared from the livers of male rats by homogenizing the liver in 0·25M-sucrose containing disodium ethylenediaminetetraacetate (2%, w/v) and fractionating with a Spinco ultracentrifuge (Model L) in the usual way. The microsomes from 1 g. wet liver were suspended in 10 ml. of a solution containing Krebs–Ringer phosphate solution (pH 7·3, 8 ml.), nicotinamide (1·6M, 1·0 ml.), NADP (0·2 mM, 1·0 ml.), glucose 6-phosphate (0·1M, 1·0 ml.) and glucose-6-phosphate dehydrogenase (130 μg. in glycylglycine buffer, pH 7·4, 0·02M, 50 μl.). Each incubation was carried out on a mechanical shaker at 37° by using 1·9 ml. of the above suspension which had been gassed while at room temperature for 10 sec. with nitrogen, and to which 0·1 ml. of a dioxan solution of the steroid (60–200 μg.) had been added immediately before gassing. The suspensions were extracted after incubation for 5, 15 and 45 min. with dichloromethane–ethyl acetate (1:1, v/v, twice with 10 ml.). After cleaning up in the usual way, the extracts were run on paper chromatograms and estimated by scanning the strips after treatment with alkaline blue tetrazolium (α-ketols) or alkaline *m*-dinitrobenzene (17-oxo steroids) (Bush, 1960).

The initial rates of reduction of 9α-chlorocortisone and of 9α-chloroandrost-4-ene-3,11,17-trione were approximately three times, and of 9α-fluorocortisone and of 9α-fluoroandrost-4-ene-3,11,17-trione approximately six times, as large as the rates of reduction of the related non-halogenated steroids. These results are in accord with the stereochemical theory of Bush & Mahesh (1959).

We are extremely grateful to the Rockefeller Foundation, the Nuffield Foundation, and the Medical Research Council for financial support of various parts of this work.

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Studies on Ribosomal Proteins from Reticulocytes

By P. Cohn.* (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W.* 3)

Extraction with dilute hydrochloric acid of microsomal ribonucleoprotein fractions obtained from rat liver (Cohn & Butler, 1958) had revealed the presence of basic protein in them (Butler, Cohn & Simson, 1960). These proteins were found to differ from the total ribonucleoprotein (RNP) in the proportion of the basic amino acids (arginine + histidine + lysine = 25 moles/100 moles of amino acids recovered in acid-soluble proteins, 19 moles in RNP) and of the principal *N*-terminal amino acids (alanine, glycine, proline) (Cohn, Simson & Butler, 1960). Since ribosomes are known to be a main site of protein biosynthesis, it was thought possible that a study of proteins of ribosomes from a cell that specializes in the synthesis of one protein only would yield requisite information on some fundamental properties of ribosomal proteins.

In the present investigation ribosomes of reticulocytes from rabbits made anaemic by injection of phenylhydrazine were obtained by differential centrifugation after lysis of the cells (Schweet, Lamfrom & Allen, 1958). Treatment of ribosomes with 0·2N-HCl at 2° for 90 min. extracted 40–45%

of the protein but very little RNA. These extracted proteins were found to be somewhat richer in basic amino acids (24 moles/100 moles) than the total protein of the ribosomes (21·3 moles/100 moles) (Tso, Bonner & Dintzis, 1958). Alanine, glycine, proline and serine accounted for at least 74% of the *N*-terminal groups found in the proteins of both the acid extract and the residue. There were, however, marked differences in the abundance of these four amino acid residues: alanine 22% in extract, 8% in residue; glycine 14% in extract, 40% in residue. The same end groups had previously been detected in the basic proteins of microsomes from rat liver (Cohn *et al.* 1960).

Electrophoresis in starch-gel prepared in 0·02N-HCl–6M-urea resolved the acid-soluble proteins into 14 to 16 bands. When reticulocyte ribosomes had been extracted with guanidinium chloride (Cox & Arnstein, 1962) and the proteins so obtained were submitted to electrophoresis under the same conditions a pattern of bands could be seen which contained some of those yielded by the acid-soluble proteins alone. It was observed that the pattern of the acid-soluble proteins differed only in some detail from that given by the basic proteins extracted from ribosomes prepared by the action of sodium deoxycholate on microsomes from rat liver.

The results obtained would appear to suggest that many of the protein components of ribosomes from rabbit reticulocytes and rat liver are similar.

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Intermediates in the Biosynthesis of Fatty Acids by Cell-Free Preparations of Lactating-Rat Mammary Gland

By J. Coniglio† and G. Popják.* (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

The major route of biosynthesis of fatty acids from [^{14}C]acetate in extracts of lactating mammary glands was established as the malonyl-CoA pathway on the basis of the cofactor requirements (CoA, ATP, $NADPH_2$, HCO_3^-, Mn^{2+} and biotin) of the enzyme system (Dils & Popják, 1962). We have substantiated these observations by the identification of an alkali-labile derivative of [^{14}C]malonic acid synthesized by soluble supernatants of rat-mammary-gland homogenates (Dils & Popják, 1962) incubated with ATP, CoA, Mn^{2+} and either [^{14}C]acetate plus HCO_3^- or unlabelled acetate plus $H^{14}CO_3^-$. Acetate and CoA could be replaced by acetyl-CoA in the system. After alkaline hydrolysis and acidification of the incubation mixtures the solutions were saturated with NH_4Cl and extracted repeatedly with diethyl ether. Total radioactivity derived from $H^{14}CO_3^-$ was determined on an aliquot of the ether extract. It was shown by gas–liquid radiochromatography (Popják, Lowe, Moore, Brown & Smith, 1959) and paper chromatography (Lynen, 1959) of the ether extracts that most of the fixed ^{14}C was associated with malonic acid. Although malonyl-CoA as such was not isolated we assume that the [^{14}C]malonic acid came, after the alkaline hydrolysis, from this derivative as ATP and acetate plus CoA, or ATP plus acetyl-CoA, and $H^{14}CO_3^-$ were needed for its formation.

The possible role of free malonate in the synthesis of malonyl-CoA was investigated because of its known stimulating effect on the biosynthesis of fatty acids (Popják & Tietz, 1955). Dils & Popják (1962) observed that free malonate was not incorporated into the synthesized fatty acids and suggested that it might act by suppressing the deacylation of malonyl-CoA or by inhibiting a reversible decarboxylation of malonyl-CoA. Results of our experiments with $H^{14}CO_3^-$ in the presence and absence of malonate furnished evidence for the latter hypothesis as the omission of malonate from the incubations decreased the incorporation of ^{14}C into malonyl-CoA to a value as low as 15% of that obtained in the presence of malonate. Substitution of other carboxylic acids (oxaloacetate, succinate, citrate or oxoglutarate) for free malonate resulted also in an increased incorporation from $H^{14}CO_3^-$ into compounds which were ether-extractable after alkaline hydrolysis and acidification. However, only a portion of this activity was located in the malonic acid.

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† On leave of absence from the Department of Biochemistry, Vanderbilt University, Nashville, Tenn., and holder of a U.S.A. Public Health Service Special Research Fellowship.

The Incorporation of Labelled Amino Acids into Subcellular Fractions from Brewer's Yeast

By A. H. Cooper.* (*The Brewing Industry Research Foundation, Nutfield, Surrey*)

In connexion with investigations into the mechanism of protein synthesis by brewer's yeast, selected strains of *Saccharomyces cerevisiae* (British National Collection of Yeast Cultures nos. 239 and 240) grown for varying times in malt extract–yeast extract–glucose–peptone medium were disintegrated by shaking for 10–15 min. with Ballotini beads (0·25 mm. diameter) in a Mickle disintegrator. The homogenates were freed from unbroken cells and coarse cellular debris by centrifugation at 1000*g* and the supernatant fluids divided into three fractions by centrifugation at 20 000*g* and 130 000*g*. Examination in the ultracentrifuge of the fraction sedimenting at 130 000*g*, which was rich in microsomes, showed that the major component had a sedimentation coefficient of 80s, although the proportions and concentrations of other components depended both on the nature of the extraction medium and on the physiological state of the yeast. Only in exponentially growing cultures, including those undergoing synchronous division, were the 80s particles predominant. Cells in the late logarithmic phase of growth, or those disintegrated in, for example, 0·05M-phosphate buffer solution at pH 7·5, showed considerable proportions of 60s and 40s components, together with lesser amounts of other components.

When exponentially growing yeast cells were shaken at 25° in the growth medium containing ^{14}C-labelled algal protein hydrolysate, a significant uptake of labelled amino acids by the microsomal fraction occurred. This was, however, considerably exceeded by incorporation into the 20 000*g* fraction, which contains as yet undefined cell fragments but which is presumably rich in mitochondria. These results recall similar observations with mitochondria from a number of different sources, and indicate that the relative roles of the various cell fragments in protein synthesis require reappraisal. Ultimately the cell sap received the greatest proportion of the total labelling. In corresponding cell homogenates, the incorporation of labelling by the 20 000*g* fraction was again far greater than that by the microsomal fraction but comparatively little radioactivity was released to the cell sap. Radioactivity was incorporated into peptidyl-nucleotidates, which were extracted from both the microsomal and the mitochondrial fractions and were present also in the cell sap. It appears therefore that, under the conditions used, a large part of the protein synthesis in the whole cell resides in material which is ultimately found in the 20 000*g* fraction. In the cell homogenates almost all the protein synthesis obtained under our conditions is localized in this heavier fraction, the microsomes having little activity. In these circumstances, however, the cell sap received little labelling.

The Electrophoretic Separation of Cellular Constituents

By J. B. Davenport.*† (*Low Temperature Research Station, University of Cambridge*)

Electrophoresis in a viscosity gradient (zonal density-gradient electrophoresis) is a suitable technique for the separation of membranous fragments resulting from the homogenization of cells (Svensson, 1960). That such separations are possible depends on the basic assumption that different structures in the cell, with their characteristic enzymic activities, have chemical compositions differing from each other and resulting in differing surface charge densities. Provided that certain physicochemical conditions are fulfilled the particles will migrate in an electric field at a rate depending only on their zeta potential and independently of their size. The factors governing the choice of electrolyte and nature of the gradient will be discussed.

The separations were carried out in the apparatus, with slight modifications, described by Partridge & Elsden (1961). The electrolyte was 0·01M-tris–0·005M-EDTA, pH 7·6. Descending electrophoresis in sucrose gradients was used, the gradient being 'tailored' to achieve adequate separation and, where possible, to preserve reasonable osmotic conditions. The most serious practical difficulty was droplet sedimentation but this may be eliminated by careful adherance to the principles of zone stability derived by Svennson (1958) and by the use of three coupled vessels for introduction of the sample and formation of the gradient as suggested by this author. The automatic introduction of sample and formation of gradient yields reproducible results.

All the particulate matter from liver and muscle which has been examined so far bears a net negative charge under these conditions and thus migrates towards the anode. A theoretical approach to electrophoretic migration through a medium of increasing viscosity will be presented which allows an approximate estimation of the zeta potential to be made.

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The method has been applied to a 'mitochondrial' (600*g*/10 min.–10 000*g*/30 min.) fraction and a 'microsomal' (10 000*g*/30 min.–100 000*g*/120 min.) fraction from rat liver. After electrophoresis at 1° for 18 hr. in a field strength of 2·5 v/cm., constant-volume fractions from the column were assayed for protein, succinic dehydrogenase, glucose 6-phosphatase, adenosine triphosphatase and acid phosphatase activities. After solubilization of the particles with cetyltrimethylammonium bromide the ratios of the extinction at 260 mμ to that at 280 mμ and at 400 mμ to that at 350 mμ were determined. Both fractions were heterogeneous. The 'mitochondrial' fraction separated into at least 5 components and the 'microsomal' fraction into at least six components. Components with similar composition, enzymic activity and mobility appeared in both fractions suggesting that some membranous structures fragment on homogenization to particles of widely differing size.

The technique shows great versatility as the mobilities can be altered at will by changing either the pH, the nature and concentration of the ions present or the nature and shape of the gradient.

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Fatty Acid Esterification in Lactating-Rat Mammary Gland

By R. Dils and B. Clark. (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham* 15)

Recent studies have demonstrated two distinct pathways for the incorporation of fatty acids into the glycerides of a number of tissues (see Hübscher (1961) for review). One pathway involves L-α-glycerophosphate as precursor, with phosphatidic acid and D-αβ-diglyceride as intermediates; the other is the direct acylation of monoglycerides to di- and triglycerides. Both pathways require the prior activation of fatty acids by ATP and CoA.

Fatty acid biosynthesis from acetate occurs predominantly in particulate-free fractions of homogenates of lactating rate mammary glands (Dils & Popják, 1961, 1962; Abraham, Matthes & Chaikoff, 1961). The incorporation of [1-^{14}C]palmitic acid into the glycerides of this tissue has been investigated by the method of Clark & Hübscher (1961) to determine the mechanism and intracellular site of fatty acid esterification.

With homogenates in the presence of ATP (6·6 mM), CoA (0·16 mM), glutathione (3·3 mM), $MgCl_2$ (3·3 mM), NaF (14 mM) and [1-^{14}C]palmitic acid (0·75 μC, 0·5 mM), there was a strong requirement for α-glycerophosphate (6·6 mM), which brought about a 12- to 88-fold increase in incorporation. Specific activities up to 218 mμmoles incorporation/hr./mg. of protein have been obtained. α-Glycerophosphate could not be replaced by β-glycerophosphate, glycerol or α-mono-olein. Phosphatidic acid caused a marked decrease in the incorporation of palmitic acid in the presence of α-glycerophosphate. The main pathway of glyceride synthesis is therefore via L-α-glycerophosphate and not via monoglyceride.

Virtually no incorporation occurred in the absence of ATP, CoA or $MgCl_2$. The omission of NaF brought about a 13% decrease in activity and no increased incorporation was obtained by the further addition of CTP, UTP or GTP. The addition of 0·67 mg. of albumin per ml. brought about a 1·6- to 2·4-fold stimulation.

When homogenates were fractionated by centrifugation, only about 20% of the original activity was recovered in subcellular components when these were tested separately. Addition of particle-free supernatant to mitochondrial or microsomal fractions usually caused a marked increase in incorporation. Boiled supernatant did not have this effect and no incorporation occurred with supernatant alone.

Analysis of the glycerides synthesized by homogenates showed that 63% of the total activity was in triglycerides, the remainder being in the diglycerides. The glycerides synthesized by isolated mitochondria or microsomes showed activity equally divided between diglycerides and triglycerides.

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The Incorporation of [^{32}P]Phosphate *in vivo* into Brain Phosphoinositides

By R. B. Ellis and J. N. Hawthorne.* (*Department of Medical Biochemistry and Pharmacology, Medical School, Birmingham* 15)

It is now known that at least three inositol lipids occur in ox and rabbit brain, based respectively on

inositol mono-, di- and tri-phosphates. They are 1-phosphatidylinositol (I), 1-phosphatidyl (inositol 4-phosphate) (II), and 1-phosphatidyl (inositol 4,5-diphosphate) (III) (Dittmer & Dawson, 1961; Grado & Ballou, 1961; Ellis & Hawthorne, 1961).

The incorporation of inorganic [^{32}P] phosphate into these lipids *in vivo* has been studied. Carrier-free $Na_2H^{32}PO_4$ in isotonic saline was injected into the sub-arachnoid space in rabbits anaesthetized with Nembutal. After 6 hr. the rabbits were killed and the brains removed. The extracted brain phospholipids were hydrolysed under mild alkaline conditions and the resulting phosphate esters separated and identified by chromatography on Dowex 1 (Ellis & Hawthorne, 1961).

The results show that the specific activity (counts per mg. of lipid P) of the three phosphoinositides increases in the order I < II < III. Typical values were I, 54; II, 202; III, 306. These are, of course, average values for all the phosphorus atoms in each molecule: enzymic removal of the phosphates from the 4- and 5-positions of the inositol of III showed that these phosphates had a much higher specific activity than the diester phosphate in the 1-position. Similar results have been obtained with rat brain *in vivo* by Wagner, Lissau, Hölzl & Hörhammer (1961) and *in vitro*, with rabbit brain slices, by Brockerhoff & Ballou (1962).

Such findings are consistent with a biosynthetic scheme for compound III which involves stepwise phosphorylation of phosphatidyl inositol (I). The rapid turnover of the monoester phosphate in compounds II and III suggests a role in the transport of cations in nervous tissue.

Experiments are in progress to attempt to obtain direct evidence for this.

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The Collagenolytic Activity of Rat-Liver Lysosomes

By D. Mary Frankland and C. H. Wynn. (*Rheumatism Research Centre, University of Manchester, York Place, Manchester* 13)

It has recently been shown (Frankland & Wynn, 1961) that rat liver contains an enzyme which is capable of partially degrading collagen. With the denatured dyed collagen, Azocoll, as substrate, it was demonstrated that the enzyme activity is essentially of lysosomal origin.

These results have now been confirmed by similar experiments with undenatured acid-soluble collagen as substrate. Thus, the ability of rat liver homogenates both to reduce the viscosity of acid-soluble collagen at pH 4·0 and to release labelled fragments from acid-soluble [^{14}C]collagen is similarly localized in this subcellular particle fraction.

The formation of fibrils from acid-soluble collagen solutions has been extensively investigated by Wood & Keech (1960). A concentrated lysosomal suspension, prepared by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), was ultrasonically disintegrated and the sonicate centrifuged to remove ruptured membranes. Parallel studies of the effect of this lysosomal extract on the viscosity of acid-soluble collagen solutions and on the ability of such treated solutions to form fibrils on elevation of the pH to 7·0 at 30° at suitable ionic strengths were performed. The lysosomal extract considerably reduced the viscosity of the acid-soluble collagen when incubated at pH 4·0 at 35° for 1 hr. and the resultant incubation mixture no longer formed fibrils. This contrasts with the finding of Kühn, Kühn & Hannig (1961) that trypsin-treated acid-soluble collagen retained its ability to form fibrils.

On heating the extract to 65° for 40 min., cooling to 0° and centrifuging at 16 000*g* for 15 min., it was found to be inactive against collagen since no reduction in viscosity was observed and fibril formation was normal. These results suggest that the collagenolytic activity of the lysosomal extract is not due to cathepsin C, since this thermal inactivation procedure has been shown by Tallan, Jones & Fruton (1952) to be insufficient to denature this enzyme.

The partial inhibition of collagenolytic activity by *p*-chloromercuribenzoate and the reversal of this inhibition by cysteine have also been investigated and will be discussed.

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The Effects of Anaphylaxis on the Lipid Metabolism of Guinea-Pig Lung

By P. GOADBY and W. G. SMITH.* (*Research Laboratory in Biochemical Pharmacology, Department of Pharmacy, Sunderland Technical College, Sunderland*)

It has previously been shown that lipid is lost from guinea-pig lungs subjected to anaphylactic shock *in vitro* whilst undergoing perfusion with Tyrode solution through the pulmonary artery, and that these losses were largely prevented in lungs pretreated with anti-anaphylactic doses of ethanolamine (Smith, 1961). The following experiments were undertaken to establish whether or not similar changes occurred in guinea-pig lung as a result of anaphylaxis *in vivo*.

Following anaphylaxis induced by exposing sensitized guinea pigs to aerosolized antigen, the following changes in the lipid content of the lungs have been observed: (1) a loss of cholesterol (30% of total) and a loss of phospholipid (15% of total) during the first 15 min. after shock; (2) a replacement of the lost lipid during the subsequent 15 min. Fatty acids of the neutral-lipid and phospholipid fractions were isolated after hydrolysis, methylated, and examined by gas–liquid chromatography. Both neutral-lipid and phospholipid fractions had a normal fatty acid composition 15 min. after anaphylactic shock, but at 30 min. and 60 min. after shock definite abnormalities were noted. Neutral lipids then contained less stearate and more linoleate than normal lungs, whilst phospholipids contained more palmitate and less linoleate and linolenate than normal.

It has been concluded from these data that anaphylaxis *in vivo* causes the following changes in the lipid metabolism of guinea-pig lung: (*a*) a loss of cholesterol and phospholipid (probably as a high-density lipoprotein); (*b*) stimulation of cholesterol, glyceride and glycerophosphatide synthesis to effect replacement of the lost constituents; (*c*) replacement of a high proportion of the lung lipids with newly synthesized material of abnormal fatty acid composition.

These conclusions suggest that anaphylaxis-type reactions in sensitized tissue lead to distortions in the lipid metabolism which have previously been unsuspected and which are of sufficient magnitude to exert a profound influence on the course of disease processes having an immunological basis.

We wish to thank Mr R. Parkinson for his valuable technical assistance.

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Further Observations on the Activation of Ox-Liver L-Glutamate Dehydrogenase by Phenylmercuric Acetate

By G. D. GREVILLE and A. S. MILDVAN.† (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

The process of activation of L-glutamate dehydrogenase by phenylmercuric acetate (PMA) (Hellerman, Schellenberg & Reiss, 1958; Mildvan & Greville, 1962) at pH 8·5 is slowed by L-glutamate and NAD, also by glutarate, a competitive inhibitor of the enzymic catalysis. α-Methylglutamate and ββ′-dimethylglutarate, which are not inhibitors, do not have this effect. Hence PMA probably combines near to the active centre.

With L-glutamate as substrate, α-oxoglutarate is a competitive inhibitor (Caughey, Smiley & Hellerman, 1957); PMA increases its K_i value eightfold, although it increases $V_{max.}$ only threefold. ADP increases V_i threefold. PMA increases the K_i value of glutarate more than twentyfold. Hence PMA probably weakens the binding to the enzyme of the inhibitory product α-oxoglutarate: this may be responsible, at least partly, for the activation.

The effect of pH (0·027 M-pyrophosphate buffers) on $V_{max.}$ and K_m for glutamate has been determined at constant NAD concentration, with and without PMA (0·5 m-mole/g. of protein). Plots of $\log V_{max.}$, pK_m and $\log V_{max.}/K_m$ against pH indicate the following approx. pK values: without PMA, 7·9 and 8·5–8·8 in the free enzyme, 7·2 and 8·6–8·9 in the enzyme–substrate complex; with PMA, 7·9 in the free enzyme, 8·2 in the complex. PMA thus causes disappearance of a pK, possibly attributable to a sulphydryl group, of 8·6–8·9 and reveals a pK of 8·2, both in the enzyme–substrate complex; evidence for the disappearance of the pK of 8·5–8·8 in the free enzyme is less satisfactory.

PMA lowers the sedimentation constant of the enzyme, probably by causing disaggregation into sub-units (Mildvan & Greville, 1962). With $NADH_2$ (0·9 mM) and ATP (0·5 mM) both present the enzyme showed two distinct peaks in the ultracentrifuge (cf. Frieden, 1959*b*); these were replaced by a single peak, with S intermediate between the S values of the two peaks, when PMA (32 μmoles/g. of protein) was added as well. A single peak with similar S value was found with PMA in absence of $NADH_2$ and ATP. If the fully associated enzyme is a tetramer and excess of $NADH_2$

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dissociates it into monomers (Frieden, 1958, 1959*a*), PMA seems to promote appearance of intermediate certain forms (dimers or trimers), in contrast with halogenated phenols which cause monomer formation (Wolff, 1962).

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Steroid Biosynthesis in a Case of Testicular Feminization

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The syndrome of testicular feminization is uncommon (Morris, 1953) and little is known of steroid biosynthesis in this condition.

In the present case, cryptorchid testes were removed surgically from a 17-year-old patient. While possessing an *XY* chromosome pattern this patient displays a female phenotype. The daily excretion of 17-oxo steroids was $5{\cdot}2 \pm 2{\cdot}1$ (S.D.) mg. (range for normal 17-year-old male or female 3–9 mg./day; Hamburger, 1948) and of oestrone $1{\cdot}6 \pm 0{\cdot}8$ μg. (normal female, post-menopausal, range 0·4–5·2 μg./day; Brown, 1958; in follicular phase, range 11–31 μg./day and in luteal phase of the menstrual cycle, range 10–23 μg./day; Brown, 1955).

The testes weighing 5·1 and 6·6 g. each contained a small Sertoli-cell tumour. Minced samples of tumours (400 mg.) and of associated testicular tissue (200 mg.) were incubated separately in Krebs–Ringer bicarbonate medium with glucose and [4-^{14}C]steroids in 95% O_2:5% CO_2 at 37° for 2 hr.

The following steroids have been tentatively identified among the products of incubating [4-^{14}C]progesterone with tumours: 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-hydroxypregn-4-en-3-one, androst-4-ene-3,17-dione and testosterone. Steroid biosynthesis by human Sertoli-cell tumour tissue has therefore been confirmed. Both tumour and testicular tissue incubated with [4-^{14}C]androst-4-ene-3,17-dione gave testosterone in good yield. Thus the 'feminizing' testes possess the 17β-hydroxy steroid dehydrogenase necessary for the formation of the male hormone, testosterone.

These findings will be discussed in relation to current views on the biosynthesis of sex hormones in human subjects.

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Identification of Lysosomes in Rat Hepatic Cells by Combined Cytochemical Staining and Electron Microscopy

By S. J. Holt* and R. Marian Hicks. (*Courtauld Institute of Biochemistry, and Bland Sutton Institute of Pathology, Middlesex Hospital Medical School, London, W.* 1)

Recent studies on fractions of rat-liver homogenates have shown the presence of a special group of particles (lysosomes) which contain several acid hydrolases, none of which show activity towards their substrates unless the integrity of the lysosomal membrane is damaged (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955; de Duve, 1959). Electron-microscopical studies of lysosome fractions have shown the presence of several morphological components of the hepatic cell, such as vacuolated dense bodies, microbodies and ferritin-containing granules (Novikoff, Beaufay & de Duve, 1956; de Duve, 1960). In intact cells, these components are mainly situated in the pericanalicular cytoplasm, and all or any of them could be the morphological counterpart of the biochemically defined lysosome.

Acid phosphatase is a characteristic lysosomal enzyme for which a cytochemical staining technique has been described (Gomori, 1952). This has recently been re-examined and conditions established under which reliable results may be obtained (Holt, 1959). When the modified method is applied to rat liver, discretely stained particles are seen in the peribiliary regions of the hepatic cells. Treatment before staining with agents which release lysosomal enzymes, e.g. Triton X-100 or lecithinase, either abolish the staining reaction, or give very diffuse staining, and it seems reasonable to assume that the staining method normally

demonstrates lysosomes. The resolving power of the light microscope, however, is too poor for the morphology of the stained particles to be determined. Further modifications of the staining procedure have made it suitable for electron microscopy and when used for this purpose the only structures that stain are the vacuolated dense bodies, and it may be concluded that these are the morphological counterparts of the lysosomes of liver homogenates (Holt & Hicks, 1961). Not all vacuolated dense bodies show acid-phosphatase activity, a finding which appears to be consistent with the hypothesis (de Duve, 1959) that the lysosomes comprise a single population of particles within which variations in enzymic content are correlated with the size and density of the particles, but not with their sensitivity towards rupturing agents.

Staining of microbodies has not been seen with livers from our strain of normal rats (cf. Essner & Novikoff, 1961) and this apparent lack of acid-phosphatase activity is consistent with the suggestion (de Duve, 1960) that microbodies represent a different group of particles which contain uricase, D-amino acid oxidase and catalase (de Duve *et al.* 1960).

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Structural and Enzymic Aspects of Fat Metabolism in the Small-Intestinal Mucosa

By G. HÜBSCHER,* B. CLARK and M. E. WEBB. (*Department of Medical Biochemistry and Pharmacology, University of Birmingham, Birmingham* 15)

Glyceride formation in the small-intestinal mucosa has usually been studied with subcellular preparations such as homogenates (Dawson & Isselbacher, 1960), mitochondria (Clark & Hübscher 1961*a*, *b*; Hübscher & Clark, 1961), or microsomes (Senior & Isselbacher, 1961). A survey of the intracellular distribution of enzymes catalysing triglyceride formation has now been undertaken so that enzymic activities may eventually be correlated with histological observations.

Scrapings of cat small-intestinal mucosa were gently homogenized in 0·3M-sucrose. Solid $BaSO_4$ was added to precipitate mucus, and cell fractions were prepared by differential centrifugation.

The microsomal was the most active fraction when assayed for lauryl thiokinase (0·7–1·2 μmoles of ester formed/hr./mg. of protein) and phosphatidic acid phosphatase (2·1–2·5 μmoles of P_i liberated/hr./mg. of protein). It was also the most active fraction in the biosynthesis of higher glycerides using either α-glycerophosphate (44 μm-moles of palmitate incorporated/hr./mg. of protein), or monoglyceride (47·4 μm-moles of palmitate incorporated/hr./mg. of protein) as precursor of glyceride glycerol.

The microsomes contained over 60% of the lauryl thiokinase and phosphatidic acid phosphatase activities of the nuclei-free homogenate. Most of the remainder of these activities was in the mitochondrial fraction. Senior & Isselbacher (1960) and Johnston & Bearden (1962) found similar distributions of these enzymes in rat and hamster intestinal mucosa. The microsomal fraction contained 65% of the total biosynthetic activity when monoglyceride was the glyceride-glycerol precursor. With α-glycerophosphate as precursor, the particulate-free supernatant was required for optimum activity. In the presence of this supernatant, the microsomal fraction contained between 70 and 88% of the total activity, the remainder being in the mitochondria. The supernatant could be partly replaced by serum albumin.

These results indicate that the endoplasmic reticulum is the most important subcellular structure for glyceride biosynthesis in mucosal cells. Recent histological investigations (Palay & Karlin, 1959; Clark, 1959) show that fat droplets enter epithelial cells by pinocytosis. Once inside the cells, the membrane-surrounded droplets approach the endoplasmic reticulum through which they travel to be discharged into extracellular space.

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Oxidation and Glycolysis in Subcellular Fractions from Small-Intestinal Mucosa

By G. HÜBSCHER* and H. S. A. SHERRATT. (*Department of Medical Biochemistry and Pharmacology, University of Birmingham, Birmingham* 15)

The energy metabolism of small-intestinal mucosa is of interest in relation to absorption of food and mucosal biosynthesis of fat. A high glycolytic rate of intestinal preparations was first described by Weil-Malherbe (1938) in rats and has since been studied in several species (Rosenthal, 1947; Wilson, 1956). In the present study, the rate of formation of lactic acid is compared with the activities of oxidative enzymes of the pentose phosphate cycle and the oxidation of some citric acid-cycle intermediates.

Subcellular fractions were prepared from mucosal scrapings of the cat, guinea-pig, and rat as described by Hübscher, Clark & Webb (1962). Glycolytic activity was measured by determining the L-lactic acid produced by using crystalline lactate dehydrogenase (Pfleiderer & Dose, 1955). Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed as described by Glock & McLean (1953). Oxidation of tricarboxylic acid-cycle intermediates was measured by the Warburg technique.

The nuclei-free homogenate of cat small intestine had a high glycolytic activity (3·2 μmoles of lactate formed/hr./mg. of protein), over 90% of which was found in the supernatant. The same preparation catalysed the oxidation of 0·76 μmole of glucose 6-phosphate and of 0·73 μmole of 6-phosphogluconate/hr./mg. of protein. Again, 93–98% of the activity was in the supernatant.

The distribution of succinoxidase (as percentage total activity of nuclei-free homogenate) in subcellular fractions from cat small-intestinal mucosa was: mitochondria 77, microsomes 19 and supernatant < 2. The Q_{O_2} for mitochondrial preparations varied between 42 and 91 μl. of O_2/hr./mg. of protein, the average of six determinations being 65·6. However, the mitochondria did not esterify inorganic phosphate. Pyruvate, citrate, and α-oxoglutarate were oxidized at negligible rates.

It was confirmed that mitochondrial preparations from the small-intestinal mucosa of the rat had a very low succinoxidase activity (Q_{O_2} 0·5–4·3) (Stanbury, 1961). In contrast, mitochondrial preparations from guinea-pig small-intestinal mucosa oxidized citrate, α-oxoglutarate, succinate and pyruvate with Q_{O_2} values of 82, 95, 122 and 49 respectively. They also showed respiratory control and oxidative phosphorylation with a P:O ratio of 2·6 for citrate.

It is a pleasure to acknowledge the competent help of Miss S. Duckworth.

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An Effect *in vitro* of Hypoglycin

By H. J. YARDLEY and G. GODFREY. (*Medical Research Council Unit for Research on the Experimental Pathology of the Skin, The Medical School, Birmingham* 15)

Hypoglycin [α-amino-β-(2-methylenecylopropyl)propionic acid] (Ellington, Hassall, Plimmer & Seaforth, 1959) is a toxic constituent of the fruit of *Blighia sapida* (ackee) and the cause of Jamaican vomiting sickness (Hassall & Reyle, 1955). When administered to animals orally or by injection, its most pronounced pharmacological effect is an immediate fall in liver glycogen followed by a fall in blood sugar (Patrick, 1954). Earlier theories of its action sought to implicate interference with glycogen synthesis (Patrick, 1954; Feng & Patrick, 1958), though this could not be demonstrated *in vitro*, even with concentrations of hypoglycin as high as 10^{-2}M (Feng & Patrick, 1958).

More recently, hypoglycin has been shown to increase the fat content of liver (Chen, Anderson, McCowan & Harris, 1957) and also the non-esterified fatty acids in serum (De Renzo *et al.*, 1958). These facts, together with the discovery by Holt & Holt (1958) that riboflavin reduces the toxic effect of hypoglycin, suggest that hypoglycin may act by inhibiting oxidation of fatty acids by blocking acyl-CoA dehydrogenases, thus leading to increased utilization of glucose. There is some evidence (Yardley & Godfrey, 1961) that fatty acids derived from lecithin are an important part of the endogenous substrate of skin. The effect of hypoglycin on the endogenous respiration of skin was therefore tested.

Slices of guinea-pig ear skin were floated on Krebs–Ringer phosphate (Krebs, 1933) in micro-

respirometers (Cruickshank, 1954) at 37°. After 2 hr., hypoglycin in 0·9% sodium chloride was added to half of the cultures to make the final concentration of hypoglycin 10^{-4}M; 0·9% sodium chloride was added to the controls. The average drop in respiration rate compared with the controls in seven experiments was 14%: this difference is statistically highly significant ($P < 0·001$). When glucose (0·02M) was present in the culture medium, the average drop in respiration rate on adding hypoglycin was only 4%, a difference which is probably not significant ($0·05 < P < 0·1$).

The interference with endogenous respiration, a considerable part of which is the result of phospholipid metabolism (Yardley & Godfrey, 1961), and the fact that no interference with respiration occurs when glucose is present, suggests that hypoglycin acts by blocking fatty acid oxidation.

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Observations on the Species Specificity of Histone

By E. W. Johns.* (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3*)

The histones extracted from all tissues so far examined are quite complex, but can be separated into three main groups, characterized according to the predominant basic residue present, viz. f1, lysine-rich; f2, slightly lysine-rich; f3 arginine-rich. Preparations of these three fractions from several tissues of the calf and rat showed no significant differences when examined by total and *N*-terminal amino acid analyses and starch-gel electrophoresis (Hnilica, Johns & Butler, 1962). However, Cruft, Mauritzen & Stedman (1957) obtained a histone from wheat germ and showed that it differed in certain physicochemical properties from the histones of animal tissues. A preparative and analytical study has therefore been made of wheat-germ histone.

Wheat-germ histone was prepared by extracting the deoxyribonucleoprotein, prepared by the method of Mirsky & Pollister (1942), with 0·25N-HCl. The histone so obtained differed from that of the animal tissues in: (1) a higher total lysine content (17·6% lysine); (2) 77% of the *N*-terminal amino acids were alanine; (3) not precipitating with ammonia; (4) a different starch-gel electrophoretic pattern, having no bands in the position of the arginine-rich histones.

The last finding is surprising since in animal tissues an alanine *N*-terminal group is associated with the arginine-rich histones. Other facts indicating the probable absence, in any quantity, of arginine-rich histones similar to those obtained with animal tissues are the failure to obtain them from the wheat germ by the 80% ethanol–hydrochloric acid extraction method (Johns, Phillips, Simson & Butler, 1960) or to separate them from wheat-germ histone by chromatographic methods or fractional precipitation.

As there seems to be no reason to expect that the arginine-rich histone is lost during the preparation of the deoxyribonucleoprotein, or that it is not extracted by aqueous acid, we conclude that there is a marked difference between the histone of wheat germ and that of the animal tissues examined.

A partial fractionation of the wheat-germ histone has been achieved by precipitation in stages with acetone.

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How Many Malate Dehydrogenases in Rat-Brain Homogenate?

By M. K. Johnson. (*Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey*)

A bimodal subcellular distribution for malate dehydrogenase has been reported for various tissues (Christie & Judah, 1953; Delbrück, Schimassek, Bartsch & Bücher, 1959; Siegel & Englard, 1960; Englard, Siegel & Breiger, 1960). Spectrophotometric assay of both forward and back reaction show a more complicated picture in rat-brain

homogenates. 45% is in the supernatant and 40% in the mitochondrial fraction, but about 20% of the particulate enzyme is released by suspending in water, although washing with sucrose–magnesium chloride solutions had no effect. The remaining particulate enzyme is firmly bound.

It seems unlikely that the supernatant enzyme represents leakage from damaged mitochondria. However, if the soluble enzyme is considered separately, then the distribution pattern of the enzyme (mainly in the supernatant fraction with a small amount readily liberated into solution from the mitochondrial fraction by hypotonic treatment) is identical to that demonstrated for all the glycolytic enzymes except hexokinase (Johnson, 1960), and for glucose 6-phosphate and 6-phosphogluconic acid dehydrogenases (Yamada & Shimazono, 1961). Johnson (1960) suggested this could indicate the presence of cytoplasm entrapped in pieces of axon incompletely broken during homogenization. Gray & Whittaker (1962) have provided electron-micrographic evidence for the survival of pinched-off nerve-endings in the mitochondrial fraction from guinea-pig cerebral hemispheres. These nerve-endings would be expected to contain cytoplasm, and refined density-gradient separations of brain-mitochondrial preparations have shown very similar distributions of nerve-endings characterized by bound acetylcholine and of entrapped cytoplasm characterized by the presence of lactate dehydrogenase (M. K. Johnson & V. P. Whittaker, to be published).

It is concluded that the homogenate contains one particulate and one soluble malate dehydrogenase, but that the distribution pattern is confused by the difficulty of rupturing axons while not damaging intracellular particles.

The two enzymes differed only slightly in their sensitivity to inhibition by oxaloacetate above 10^{-4}M concentration. In order to study this effect in crude tissue preparations, it is necessary to add purified lactate dehydrogenase to both sample and control to remove pyruvate which is formed from the oxaloacetate, otherwise lactate dehydrogenase activity in the tissue may be measured also.

The reaction rate of the enzymes with nicotinamide–adenine dinucleotide and malate is linear and fairly rapid at pH 10, but slower and decaying at pH 8·5. The back reaction is fast and linear at pH 7·5.

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Studies on the Dinitrophenyl Derivative of a Bovine-Tracheal Chondropeptide Isolated by Papain Digestion

By P. W. Kent, Freda K. Hartley and J. M. Ashworth. (*Department of Biochemistry, University of Oxford*)

There is considerable evidence that *in situ* chondroitin sulphate is strongly associated with polypeptide material and several views have been expressed about the possible nature of the attachment (Mathews & Lozaityte, 1958; Malavista & Schubert, 1958; Muir, 1956, 1958; Partridge & Davies, 1958; Gregory & Rodén, 1961). We find that a chondropeptide fraction can be readily obtained from powdered bovine-tracheal cartilage by incubation for 48 hr. at 37° with crystalline papain (1%, w/v), cysteine (5 mM) and EDTA (1 mM) in sodium citrate buffer (0·08M) at pH 5. After dialysis (4 days, 2°) of the soluble products, the chondropeptide was separated by addition of cetylpyridinium chloride (CPC) in acidic conditions (pH 2) (Scott, 1960). The precipitated complex was dissociated in sodium chloride (2M) and digested mucosubstance was isolated by ethanolic precipitation, dialysis and freeze-drying. The resulting fraction (5·6% of initial cartilage powder, 3·4% of N; 11·1% of ester sulphate, 20·3% of ash) was homogenous in zone electrophoresis (at four pH values) and in $MgCl_2$–CPC fractionation on paper (Marsden & Kent, 1962). This chondropeptide fraction reacted (18 hr., 18°) with 1,fluoro-2,4-dinitrobenzene in 0·5M-sodium phosphate buffer (pH 7·8) containing 5% (v/v) of ethanol (cf. Pasternak & Kent, 1952). Dialysis and re-isolation with CPC in acidic conditions, as before, gave DNP-chondropeptide in 78% yield (3·8% of N; 10·2% of ester sulphate, E 0·355 at $\lambda_{max.}$ 3500 Å for 0·063% in 1% sodium hydrogen carbonate), which moved as a single band, in zone electrophoresis in a variety of buffers; on a CPC-gradient column (Antonopoulos, Borelius, Gardell, Hamnström & Scott, 1961) and in $MgCl_2$–CPC fractionation on paper. The amino sugar constituent released by acidic hydrolysis was identified (Gardell, 1953) as galactosamine (28·2%). At the same time, at least five DNP-amino acids were formed, provisionally identified (Biserte, Holleman, Holleman-Dehore & Sautière, 1959) as DNP-

glutamic (or aspartic) acid, DNP-serine, DNP-valine, DNP-alanine, DNP-leucine.

The DNP-chondropeptide, degraded in alkaline conditions (0·5N-NaOH, 25°, 40 hr.) gave typical chondroitin sulphate with release of peptide constituents which underwent simultaneous extensive decomposition.

When the DNP-chondropeptide was examined in 0·1N-NaCl in the Spinco ultracentrifuge at 15°, it was found to move as a single component, S_{15} 1·16S. The molecular weights of three different preparations at different electrolyte concentrations were in the range 21×10^3 to 25×10^3. The molecular weight calculated spectroscopically from the DNP-content was 25×10^3.

It appears likely therefore that the chondropeptide is comprised mainly of chondroitin sulphate to which peptide(s) (approx. 12%) is attached, and that on average each molecule carries one NH_2-terminal amino acid.

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Isolation of Hormone-Containing Particles from the Neurohypophysis of the Cod (*Gadus morrhua*)

By K. Lederis† (introduced by H. Heller). (*Anatomisches Institut der Universität Kiel, Kiel, Germany*)

Electron-microscopical studies of the fine structure of the neurohypophysis have shown that the axons of the supraoptic and paraventricular nuclei of the mammalian hypothalamus (or the preoptic nucleus of lower vertebrates) end in the posterior pituitary in terminal dilatations (Palay, 1957; Bargmann, 1958). The nerve terminals contain small vesicles about 1500 Å in diameter. The electron-dense centres of these vesicles have been shown to be identical with the neurosecretory substance as seen under the light microscope (Palay, 1955); the neurosecretory vesicles were assumed to contain the neurohypophysial hormones.

Lederis & Heller (1960) isolated a particulate fraction from homogenates of the rabbit neural lobe by differential centrifugation and showed electron microscopically that the fraction which contained 50–70% of the total intraparticulate hormones consisted mainly of particles identical in size and structure with the neurosecretory vesicles seen in the gland *in situ*.

The present report deals with attempts to effect the isolation and identification of hormone-containing particles from a marine teleost, the cod. It is known that the active principles of the neurohypophysis of fishes are chemically and pharmacologically different from the mammalian hormones (Pickering & Heller, 1959; Heller & Pickering, 1961). The fine structure of the fish neurohypophysis is much like that of the higher vertebrates, but it was not known in which cell particles the hormones are stored (Bargmann & Knoop, 1960).

The neurohypophyses of 15–20 freshly caught cod were collected on ice, homogenized in 0·44M-sucrose, and cell fractions obtained by differential centrifugation at 0°: 1000*g* (10 min.)—sediment = Fraction I; 9000*g* (10 min.)—sediment = Fraction II; 36 000*g* (10 min.)—sediment = Fraction III; final supernatant = Fraction IV.

The sediments were resuspended in 0·44M-sucrose, aliquots were collected for electron microscopy and the remainder was extracted for bioassay. Hormonal activity was estimated in all fractions on the blood pressure of the rat by using an extract of mammalian posterior pituitary as standard. The three sediments contained up to 80% of the pressor activity of the homogenate. Fraction III contained 49–64% of the total sedimentable hormones. Electron-microscopical examination showed that Fraction II consisted of mitochondria, mitochondrial fragments and unbroken nerve terminals with neurosecretory vesicles within. Fraction III contained mainly smaller particles the bulk of which could be identified as the neurosecretory vesicles. It is tentatively concluded, therefore, that the hormones of the fish posterior pituitary are stored in the neurosecretory vesicles.

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Intracellular Distribution and Properties of Rat-Liver Maltases

By N. Lejeune, D. G. Thines-Sempoux and H. G. Hers. (*Department of Physiological Chemistry, University of Louvain, Louvain, Belgium*)

In relation with the recent description of the presence of a maltase (α-glucosidase) in human tissues and of its absence in the cardiomegalic type of glycogen storage disease (Hers, 1961), the properties and localization of the rat-liver maltases have been investigated. Rat-liver homogenates were prepared in 0·25M-sucrose or mannitol and fractionated according to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Maltase activity was measured by the formation of glucose from maltose and its distribution pattern was compared with those of acid phosphatase, cytochrome oxidase and glucose 6-phosphatase, which served as reference enzymes for lysosomes, mitochondria and microsomes respectively. When measured at pH 3·5, maltase activity is most concentrated in the light-mitochondrial fraction and its distribution parallels that of acid phosphatase. When measured at pH 8·5, it is mostly found in the microsomes and follows glucose 6-phosphatase.

The parallelism between the acid maltase and acid phosphatase has been further demonstrated by fractionation of a light mitochondrial fraction by density equilibration in a density gradient according to Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959). In a freshly prepared particulate fraction, acid maltase shows only 10% of its potential activity; it is simultaneously activated and solubilized by freezing and thawing, by treatment with Triton X-100 or digitonin and by exposure to a hypotonic medium under the conditions described by Sellinger, Beaufay, Jacques, Doyen & de Duve (1960). In all cases, its release parallels that of acid phosphatase. These results indicate that it is most probably bound to lysosomes.

The human-liver maltase shows the same pH–activity curve as the rat-liver-lysosomal maltase. Preliminary results indicate that its distribution in human liver parallels that of acid phosphatase.

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Electrophoresis of Microsomal Components

By U. E. Loening. (*Department of Botany, University of Edinburgh*)

It has been found that intact ribosomes prepared from pea-seedling roots (Loening, 1961) migrate as sharp bands during electrophoresis in cellulose acetate membrane by the method of Kohn (1958). Ribosomes and other RNA components can be separated from membranous materials.

Membrane strips 10 cm. long and 5 cm. wide were used, and three adjacent samples run on each. 120 v was applied for about 3 hr. at 0°; the current varied from 0·6 to 1·2 mA, depending on the buffer used. The progress of the electrophoresis was followed with a Hanovia u.v. lamp emitting at 254 mμ; RNA-containing materials then appear as dark bands on a blue fluorescent background.

In all buffers tried, the membranous and phospholipid components of the microsomes remained on the origin, and the ribosomes travelled toward the anode. In 50 mM-tris–HCl buffer at pH 7·4 the mobility of the ribosomes is dependant on the Mg^{2+} concentration, from 10 to 12 mm. per hour at 0·1 mM-Mg^{2+} to less than 2 mm. per hour at above 1 mM. In 50 mM-tris, 33 mM-citric acid, pH 7·4, the mobility is about 15 mm. per hour and a u.v.-absorbing band travels ahead of the ribosomes. This band is most pronounced in membranous fractions. Low Mg^{2+} concentrations and citrate buffers favour the separation of ribosomes from whole microsomes.

The membranes can be cleared in liquid paraffin or better in Ondina Oil 17 (Scottish Oils and Shell-Mex, Ltd.) and scanned for nucleic acid components at 260 mμ. The extinction of the membrane is then 0·2–0·5, varying with the buffer present before drying. The absorption spectrum of the nucleic acids is very similar in the oil-cleared membrane to

that in buffer. The oil can be removed with light petroleum.

The membranes can also be cleared in a scintillation fluid of suitable refractive index (e.g. in trimethylbenzene solvent) and scanned for radioactivity, including ^{14}C and ^{3}H. A simple automatic scanner to fit into the sample chamber of the Ekco N664A scintillation counter has been designed for this. In order to remove acid-soluble substances, the membranes were washed in 0·5N-perchloric acid at 0° and most of the acid removed with tris buffer before drying.

The method provides a simple and rapid means for the study of the incorporation of isotopes into the different microsomal components.

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Polysaccharides of the Green Seaweed *Codium fragile*

By J. Love and Elizabeth Percival.* (*Department of Chemistry, University of Edinburgh*)

The present investigations describe the separation of three different polysaccharides from *Codium fragile*, viz. a water-soluble sulphated arabogalactan, a starch-type polysaccharide, and a pure mannan.

Preliminary chromatographic analysis of an acid hydrolysate of the whole weed revealed the presence of mannose and galactose with smaller amounts of arabinose and xylose and trace amounts of glucose. Large-scale extraction of the dried seaweed (after removal of colouring matter with acetone) with cold and with hot water was followed by extraction with zinc chloride (Iriki & Miwa, 1960) and with 4 and 20% sodium hydroxide solutions after mild chlorite treatment (Mackie & Percival, 1959). Paper chromatography of hydrolysates of the crude cold- and hot-water extracts (approx. 10% of dry weight of weed) revealed that both extracts contained mainly galactose and arabinose.

The cold-water extract gave no colour with iodine, was unattacked by salivary α-amylase and resisted fractionation by the usual reagents (Mackie & Percival, 1961). However, separation of a sulphated arabogalactan (approx. 5% of dry weight of weed) was achieved by gradient elution with increasing concentration of potassium chloride on a DEAE-cellulose column. It had $[\alpha]_D + 37·5°$, SO_3, 12·7%, gave a positive test for 3,6-anhydrogalactose, and an infrared spectrum which had the characteristic peak for axial sulphate. An acid hydrolysate contained only arabinose and galactose in the approximate molar ratio of 1:3. Treatment with alkali (Rees, 1961) reduced the sulphate content of this polysaccharide by approx. 4%, and in the residual arabogalactan (isolated in approx. 90% yield) the proportion of 3,6-anhydrogalactose had apparently risen from approx. 1 to 3% (Yaphe, 1960). It appears that the alkali had cleaved ester sulphate from $C_{(6)}$ in the galactose units with concomitant formation of the 3,6-anhydro sugar (Duff & Percival, 1941).

The material extracted with hot water gave a strong blue colour with iodine. Extraction of the cold water extracted weed overnight with hot 0·3% ammonium carbonate under nitrogen and formation of the iodine complex from this solution (Steiner & Guthrie, 1944) gave a pure starch (900 mg. from 100 g. of weed). It stained blue with iodine, had a high positive rotation, and gave only glucose on hydrolysis. It was separated (Haworth, Peat & Sagrott, 1946) into amylose (approx. 16%) and amylopectin.

Mannans, highly contaminated with silica and with small quantities of the water-soluble polysaccharides, were isolated from the zinc chloride and 4% alkaline extracts. From the 20% sodium hydroxide extract a silica-free mannan was separated and purified by precipitation as the copper complex (Jones, 1950). The pure mannan, $[\alpha]_D - 41°$, consumed 0·8 mole of periodate (Aspinall & Ferrier, 1957) for every anhydro-residue, indicating the presence of an essentially β-1→4′-linked polysaccharide in agreement with the results of Iriki & Miwa (1960). This structure was confirmed by the isolation of crystalline mannose, β-1→4′-linked mannobiose and -triose after enzymic hydrolysis of the mannan with hemicellulase (Painter, 1959; Bishop & Perila, 1961). The total mannan extracted by these methods corresponds to approx. 20% of the dry weight of weed and the residual polysaccharide remaining in the weed contains only mannose units.

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Polynucleotidic Polyisoprene Particles

By A. I. McMullen. (*The Natural Rubber Producers' Research Laboratory, Welwyn Garden City, Herts.*)

When fresh latex from *Hevea brasiliensis* is centrifuged at 53 000*g* for 40 min. most of the ribosomal particles are sedimented but are released from this sediment by treatment with deoxycholate.

They are then sedimentable by centrifugation at 170 000*g* for 2 hr. and when further washed with tris–magnetism acetate buffer give a translucent deposit which, on drying, contains 25% (w/w) of RNA. On homogenization of this with 0·1% sodium dodecyl sulphate solution and immediate recentrifugation at 170 000*g* for 3 hr. a sediment containing 31% of (w/w) of RNA is obtained together with a low-density particulate material of average diameter about 300 Å which travels centripetally. This is shown by infrared analysis to be mainly *cis*-polyisoprene containing some polar material, and on heating this suspension in 10% NaCl for an hour ultraviolet-absorbing substances are liberated. Precipitation with 70% ethanol gives a substance with typical nucleic acid u.v.-absorption characteristics:

$\lambda_{max.} = 260$ mμ, $\lambda_{min.} = 237$ mμ, $E_{max.}/E_{min.} = 1{\cdot}8$.

Repetitive washing of the original cream layer obtained on centrifuging fresh latex gradually releases sedimentable particles containing RNA which are removed by washing to an extent (representing a dilution factor of greater than $1:10^7$) where the rubber particles show signs of coalescence. When at this stage the cream layer is heated in 10% NaCl the rubber coagulates, leaving in solution u.v.-absorbing material, as before. The spectroscopic data indicate protein and nucleotide components in quantity just sufficient (0·6%, w/w, on the rubber) to cover with a monomolecular layer the available rubber surface which consists, at the final washed stage, of particles of average diameter 700 Å. This nucleoprotein contains 6% of RNA and may be reprecipitated with 70% ethanol to give nucleoprotein of considerably higher RNA content.

Thus a polyisoprene–nucleic acid association exists in latex in the form of ultra-micro particles which may be found either in the sediment or cream layer on centrifugation, depending on the ratio of rubber to 'nucleoprotein'. As will be described, such particles, together with soluble components of the latex serum, are capable of incorporating isopentenyl pyrophosphate into the rubber by a process which is strongly inhibited in the presence of ribonuclease. Thus they appear to form an essential part of an 'isopentenyl pyrophosphate polymerase' system and may be regarded as 'rubber ribosomes'. Their behaviour is consistent with a polynucleotide template theory of rubber biosynthesis (McMullen, 1961).

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Periodate Oxidation of a Purified Glycopeptide Fraction from Egg Albumin

By G. S. Marks, R. D. Marshall and A. Neuberger. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2*)

The use of periodate oxidation for the study of the oligosaccharide moiety in egg albumin (Bragg & Hough, 1961; Lee & Montgomery, 1961) has been of limited value because of interference due to protein. In the present study we prevented this difficulty by working with a purified glycopeptide fraction from egg albumin. This enabled us to measure periodate uptake, formic acid liberation and to determine which monosaccharide units were attacked by periodate.

One glycopeptide unit (containing 5 mannose and 3 glucosamine residues) was found to reduce 5·05 molecules of sodium metaperiodate and yield 0·7 molecule of formic acid. The presence of *N*-acetyl groups on the glucosamine residues prevents the liberation of formic acid on periodate oxidation (Hough & Taha, 1956). Consequently the formic acid liberated from the glycopeptide must be derived from a 1→6-linked mannose residue or a non-reducing mannose end group. After treatment of the glycopeptide with almond emulsin, mannose was detected by paper chromatography showing that a mannose residue occurs as a non-reducing end group and is the source of the formic acid liberated. This is in accord with the identification of 2,3,4,6-tetra-*O*-methyl-D-mannose amongst the products of exhaustive methylation of egg albumin in the experiments of Bragg & Hough (1961).

To determine the number of mannose residues in the glycopeptide which had survived periodate oxidation a recently developed method involving the radioisotope dilution of radioactive mannose (François, Marshall & Neuberger, 1962) was used. By this method it was shown that 53% of the mannose was lost during periodate oxidation. This compares with losses of 51 and 69% found by other workers (Bragg & Hough, 1961; Lee & Montgomery, 1961). With the method of Rondle & Morgan (1955) it was shown that 1 of the 3 *N*-acetylglucosamine residues of the glycopeptide was attacked by periodate, an observation also reported by other workers (Bragg & Hough, 1961; Lee & Montgomery, 1961).

Since 2·7 of the 5 mannose residues in a glycopeptide unit are attacked by periodate and 1 mannose is a non-reducing end group it follows that 3·7 molecules of periodate will be reduced by mannose. The *N*-acetylglucosamine would account for the reduction of 1 molecule of periodate so that 1 glycopeptide unit would be expected to reduce 4·7 molecules of periodate. This value is in fair agreement with the finding that 1 glycopeptide unit reduced 5·05 molecules of periodate.

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The Subcellular Distribution of Histamine in Guinea-Pig Brain

By I. A. Michaelson† and V. P. Whittaker. (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

Homogenates of guinea-pig brain in 0·32M-sucrose were fractionated by differential centrifugation as described by Whittaker (1959). The histamine of the homogenate and fractions was determined spectrofluorometrically by the method of Shore, Burkhalter & Cohn (1959). About 60% of the brain histamine was recovered in the low speed (P_1) fraction collected after centrifuging at $1000g \times 11$ min. and containing nuclei, myelin fragments, blood cells and tissue debris (Gray & Whittaker, 1962). Most of the remainder was recovered in the intermediate (P_2) fraction ($15\,000g \times 60$ min.) containing small myelin fragments, nerve-endings, mitochondria and membrane fragments. Relatively little was recovered in the microsomal (P_3) fraction ($105\,000g \times 60$ min.) or soluble supernatant (S_3).

The subcellular distribution of histamine in the guinea-pig brain is thus different from that of acetylcholine (Whittaker, 1959) and hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1962), the particulate-bound fractions of which are recovered mainly in the P_2 fraction.

Experiments with mast cells obtained by peritoneal lavage from the rat showed that such cells were not completely broken up under the relatively mild conditions of homogenization used for brain tissue and that they and the histamine contained in them were recovered almost entirely in the P_1 fraction. This suggests that the brain histamine recovered in this fraction may represent brain mast-cell histamine. Experiments are now under way to elucidate the nature of the histamine in the P_2 fraction which may represent the fraction of brain histamine not associated with mast cells (Adam, 1961).

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Fatty Acid Oxidation by Subcellular Particles (Mitochondria) from the Hepatopancreas of *Carcinus maenas*

By K. A. Munday and E. A. Munn.* (*Department of Physiology and Biochemistry, University of Southampton*)

There are only isolated reports on the metabolism of lipid materials by well-characterized invertebrate systems (Zebe & McShan, 1959; Meyer, Preiss & Bauer, 1960). Respiratory quotients, storage and subsequent utilization of lipids, absence of glucose 6-phosphate (Munday & Munn, 1962) and the high levels of circulatory lipids indicate that these substances may have a significant role in the metabolism of *Carcinus maenas*.

Lipids were extracted from the crab and fractionated on silicic acid into carotenoids, sterol esters, triglycerides, non-esterified fatty acids, free sterols, diglycerides, monoglycerides and phospholipids. Phospholipids represented about 50% by weight of the total lipid, cholesterol esters 30% and triglycerides 10%. The fatty acids present in these fractions were characterized by gas–liquid chromatography. The oxidation of authentic samples of certain of these fatty acids was studied *in vitro* and *in vivo*.

Subcellular respiring particles were prepared from the hepatopancreas by homogenization and differential centrifugation by the method of Munday & Thompson (1962). Their ability to oxidize fatty acids was assayed manometrically.

The conditions for the oxidation of octanoate were studied over a range of concentrations of inorganic phosphate, magnesium ions and ATP. In the presence of these cofactors the preparation oxidized octanoic acid without addition of a tricarboxylic acid-cycle intermediate. In the presence of a trace amount of succinate sparking of the fatty acid oxidation was demonstrated. With [1-^{14}C]octanoate, it has been shown that the radioactivity from the fatty acid appears in the carbon dioxide.

Palmitic, myristic, lauric, butyric and β-hydroxybutyric acids have been examined and are found to be oxidized by the mitochondrial preparation. [1-^{14}C]Octanoate and [1-^{14}C]stearate injected into the cardiac sinus of the crab were oxidized *in vivo* yielding labelled carbon dioxide.

These results show that both short- and long-chain fatty acids, representative of those occurring in the lipid reserves of *Carcinus maenas*, can be oxidized by the whole animal and by subcellular particles. The sparking phenomenon implicates the tricarboxylic acid cycle in the latter system (Munday & Thompson, 1962).

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The Acid-*p*-Nitrophenyl Phosphatase Activity of Adult Guinea-Pig Liver Subcellular Fractions

By M. W. Neil* and Marion W. Horner. (*Department of Biochemistry, The London Hospital Medical College, Whitechapel, London, E.* 1)

The simplicity of the assay procedure using *p*-nitrophenyl phosphate (PNPP) as a substrate to measure acid-phosphomonoesterase activity led to attempts in this Laboratory to use this compound to assay this activity in liver subcellular fractions.

Since hepatic glucose 6-phosphatase also hydrolyses PNPP, a routine preincubation period of 10–20 min. at 37° (acetate buffer, 0·05M or 0·10M, pH 5·0) was instituted to inactivate this enzyme (de Duve, Berthet, Hers & Dupret, 1949) for the assays. In spite of this procedure, the distribution of acid-phosphomonoesterase activity with PNPP (99–100% pure) as substrate was markedly different from that obtained with β-glycerophosphate (β-GP). The principal discrepancy was found in the much greater activity of the supernatant (S) fraction (obtained by conventional differential centrifugation of guinea-pig liver homogenized in 0·25M-sucrose) on PNPP.

Further experiments revealed that the acid-PNPPase activity of *S* was inhibited 85–90% by 0·05M-alloxan at 37° and pH 5·0. The activity of the mitochondrial (*M*) fraction (which included the lysosomal fraction) was inhibited 15–20% by this compound. (+)-Tartrate, shown to inhibit prostatic acid phosphatase by Abul-Fadl & King (1949), has the reverse effect, inhibiting the acid phosphatase of *M* by 85–90% but that of *S* by only 10–15%. The distributions of phosphomonoesterase activity in guinea-pig-liver subcellular fractions when assayed by PNPP and β-GP in the presence of 0·05M-alloxan are markedly similar.

Thus there appear to be at least three enzymes in guinea-pig liver exhibiting acid-PNPPase activity. These enzymes are glucose 6-phosphatase, as mentioned above, lysosomal acid phosphatase (LP-acid phosphatase) which is almost completely inhibited by (+)-tartrate but not by alloxan, and a third enzyme (H, hyaloplasmic acid phosphatase) which is inhibited by alloxan but not by tartrate.

Whereas the activity of LP-enzyme towards PNPP and β-GP is approximately the same, H-enzyme is considerably more active towards PNPP than β-GP. An analogous situation has been demonstrated in other animal species. The patterns of enzyme activity described above are not influenced by perfusion of the liver with ice-cold 0·25M-sucrose before homogenizing. Further characterization of H-enzyme is in progress.

The variety of the results obtained with different substrates for the assay of 'acid phosphatases' (e.g. Fishman & Davidson, 1957; Goodlad & Mills, 1957; Macdonald, 1961; Neil, 1961) suggests that reference to 'acid phosphatase' can be misleading. It is recommended that the nature of the alcoholic or phenolic moiety of the substrate should, wherever possible, be stated (e.g. acid *p*-nitrophenyl phosphatase) in order to avoid spurious comparisons.

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Specificities of Ribonucleases in Rat-Liver Cytoplasm

By J. T. Nodes, E. Reid* and J. M. Whitcutt. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3*)

With crude fractions obtained from mitochondrial preparations it was previously shown (Reid & Nodes, 1959; cf. Zytko, de Lamirande, Allard & Cantero, 1958) that alkaline ribonuclease (alkaline RNase) attacks RNA to give only pyrimidine nucleoside 2′,3′-cyclic phosphates (cyclic nucleotides) and is inactive towards a purine-rich 'core' from RNA and towards cyclic adenylic or cytidylic acid. Acid RNase fractions hydrolyse all phosphodiester bonds in RNA, and convert the cyclic nucleotides thus formed into non-cyclic nucleotides. Study of the latter process, the products being separated on Dowex 1 resin, has shown that there is rapid attack (cf. Roth, 1959) on cyclic adenylic or guanylic acid to give the 2′-isomer (optimum pH 6·3) and slow attack on cyclic cytidylic or uridylic acid to give the 3′-isomer (pH 5·7).

Mitochondrial preparations have now been fractionated on columns of modified cellulose. Mitochondria treated with deoxycholate (Roth, 1957) gave, on diethylaminoethyl-cellulose (DEAE-cellulose), multiple peaks of RNase activity. However, when the protein remaining soluble after heating at 60°, pH 3·5 (Reid & Nodes, 1959), was chromatographed, alkaline RNase was located in a peak eluted with 0·005M-phosphate (pH 7·8)–0·005M-NaCl. On rechromatography with carboxymethyl-cellulose, the activity was eluted by 0·1M-phosphate (pH 8·0)–1·0M-NaCl. The optimum pH was 7·3, and the specificity was as previously found. There was no attack on bis-(*p*-nitrophenyl) phosphate, in agreement with Roth (1959), on cyclic cytidylic acid, or, in disagreement with Roth, on cyclic uridylic acid.

The mitochondrial protein remaining soluble after heating for 5 min. at 50°, pH 5·0 (Roth, 1957), gave in the fraction eluted from DEAE-cellulose by 0·05M-phosphate (pH 6·0)–0·05M-NaCl a peak of acid-RNase activity (optimum pH 5·5) slightly contaminated with alkaline RNase. There was no activity towards either bis-(*p*-nitrophenyl) phosphate or, surprisingly, cyclic nucleotides whether purine or pyrimidine.

Roth (1959) suggested that supernatant and mitochondrial fractions contain different alkaline RNases. However, when supernatant fractions were heated at 60° and chromatographed, no difference was observed from mitochondrial fractions in the position of the alkaline-RNase peak, in pH optimum, or in specificity.

Recent observations with bovine liver by Maver & Greco (1962) in part disagree with the present findings. Their alkaline RNase was of much broader specificity, and their acid RNase slowly attacked cyclic adenylic acid giving only the 3′-isomer, the 2′-isomer being formed by a distinct enzyme.

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Zinc Complexes of Human Albumin and Poly-glutamic Acid

By D. J. Perkins.* (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W. 1*)

The binding of zinc ions to water-soluble proteins has been postulated as being through the imidazole rings of histidine residues. This postulate was based upon the ionization of the imidazole NH group at pH 6–7. No other groups ionize in that range. Patchornick, Berger & Katchalski (1957) found a 1:1 complex of zinc ions and histidine residues by using poly-L-histidine.

Perkins (1961) showed that Zn^{2+} ion binding was not related to the histidine content of human albumin and pepsin. In that study there was good reason to suppose that binding was related to the number of free carboxyl groups in the aspartic and glutamic acid residues of these proteins. The present experiments with poly-L-glutamic acid show a pH dependence which is similar to that found (Perkins, 1953) for the 1:1 zinc–histidine complex. Conclusions based on amino acid and polypeptide models and applied to proteins without direct confirmatory experiments on the protein molecules themselves are unsound.

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Stereospecificity of Hydrogen Transfer from Reduced Nicotinamide–Adenine Dinucleotides to Squalene

By G. Popják,* G. Schroepfer† and J. W. Cornforth. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12, and National Institute for Medical Research, Mill Hill, London, N.W. 7*)

It has been shown recently that during synthesis of squalene from two molecules of farnesyl pyrophosphate (FPP) one hydrogen atom attached to C-1 of one of the two FPP molecules is exchanged for a hydrogen atom derived from reduced nicotinamide–adenine dinucleotide phosphate ($NADPH_2$) (Popják, Goodman, Cornforth, Cornforth & Ryhage 1961). It was inferred from some of the results obtained that this hydrogen exchange was a stereospecific process (Popják, Cornforth, Cornforth, Ryhage & Goodman, 1962). The experiments to be reported support, at least in part, this conclusion, as they show that only one stereospecific hydrogen atom at C-4 of the nicotinamide ring of a reduced nicotinamide–adenine dinucleotide can be transferred to squalene. We have prepared 'α'-^{3}H and 'β'-^{3}H-$NADPH_2$ and $NADH_2$ by a combination of chemical and enzymic methods. [^{3}H]NADP and [^{3}H]NAD were made first by a modification of the CN-exchange method of San Pietro (1955). The [^{3}H]NADP was then reduced with isocitric dehydrogenase and glucose 6-phosphate dehydrogenase and their respective (unlabelled) substrates to give the 'β'-^{3}H and 'α'-^{3}H-$NADPH_2$. Two specimens of [^{3}H]NAD were reduced with liver alcohol dehydrogenase and with glyceraldehyde phosphate dehydrogenase giving the 'β'-^{3}H and 'α'-^{3}H-$NADH_2$ respectively. 'α'-^{3}H-$NADH_2$ was also made by the reduction of non-isotopic NAD with liver alcohol dehydrogenase and [1-$^3H_2$]3,3-dimethylallyl alcohol. The two forms of the ^{3}H-labelled reduced coenzymes were then used in the anaerobic squalene-synthetase system of rat-liver microsomes with synthetic [2-^{14}C]*trans-trans*-farnesyl pyrophosphate as substrate (Popják *et al.* 1962). It was found that only the 'β'-^{3}H-$NADPH_2$ or the 'β'-^{3}H-$NADH_2$ donated ^{3}H efficiently to squalene. Thus it may be deduced that the squalene synthetase of liver microsomes has the same steric specificity in respect of the hydrogen transferred from the reduced coenzymes as glucose 6-phosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase, designated (as yet arbitrarily) as 'β'.

† On leave of absence from Department of Physiological Chemistry, University of Minnesota, and holder of a U.S.A. Public Health Service Special Research Fellowship.

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The Effect of Quinones on the Hill Reaction Activity of Isolated Chloroplasts

By E. R. Redfearn* and J. Friend. (*Department of Biochemistry, The University of Liverpool and Department of Botany, The University of Hull*)

Plastoquinone (2,3-dimethyl-5-solanesyl-1,4-benzoquinone) functions in the photosynthetic electron transport system as an oxidation–reduction carrier, as has been shown by studies on the reactions of the endogenous material (Crane, Ehrlich & Kegel, 1960; Redfearn & Friend, 1961; Friend & Redfearn, 1962). Earlier it had been shown by Bishop (1959) that extraction of freeze-dried chloroplasts with light petroleum resulted in a loss of Hill reaction activity and that the loss of activity could be correlated with the amount of endogenous plastoquinone removed. Restoration of photolytic activity was achieved by adding plastoquinone to the extracted preparation.

In a study of the effect of quinones on the Hill reaction activity of chloroplasts before and after extraction with organic solvents, Bishop's (1959) results have been confirmed. In addition, however, it was found that a number of other quinones would also restore photolytic activity in extracted preparations and that some were more effective than plastoquinone. Furthermore, most of these quinones had marked stimulatory effects, although quantitatively smaller than the reactivating effects, on the Hill reaction activity of unextracted chloroplasts. These results raise certain problems regarding specificity and the mechanism of interaction of quinones with the photosynthetic electron-transport system.

Chloroplasts were isolated from sugar-beet or spinach leaves (Redfearn & Friend, 1961) and were extracted with light petroleum, b.p. 40–60°, without prior freeze-drying. Hill reaction activity was measured potentiometrically (Spikes, Lumry, Rieske & Marcus, 1954) with potassium ferricyanide as the electron acceptor.

The endogenous plastoquinone was removed progressively by successive extractions until the total extractable quinone had been removed. The reactivating effect of added quinones was inversely related to the amount of plastoquinone extracted.

In addition to plastoquinone, 1,4-benzoquinones with the following substituents were found to reactivate extracted preparations and to activate unextracted preparations: trimethyl- (cumoquinone); tetramethyl- (duroquinone); 2-methoxy-6-propyl-; 2,5-dimethyl-; 2-acetyl-3-ethyl-5-methoxy-. On an equimolar basis, cumoquinone and 2-methoxy-6-propylbenzoquinone were more effective than plastoquinone. Of the quinones tested related to the ubiquinone (coenzyme Q) series, aurantioglíocladin and ubiquinone-5 were active but ubiquinone-24 was inhibitory. 2-Methylnaphthoquinone (menadione or vitamin K_3) was found to be active, thus confirming the work of Bishop (1958), but 3-hydroxy-2-methylnaphthoquinone (phthiocol), vitamin K_2-5, K_2-10 and K_2-25 were inhibitory.

The results will be discussed in relation to the site and mode of action of quinones in the photosynthetic electron-transport system.

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The Intracellular Distribution of Peptidases in Rat Small-Intestinal Mucosa

By G. B. Robinson.* (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham* 15)

Newey & Smyth (1960) demonstrated that dipeptides can be absorbed intact by the epithelium of rat small intestine. Since the absorbed peptides are hydrolysed within the mucosal cells, it was of interest to determine the intracellular site of peptidase activity. In rat liver, the enzymes hydrolysing glycylglycine and triglycine (Maver & Greco, 1951) and leucinamide (Rademaker & Soons, 1957) occur mainly in the supernatant fraction.

The subcellular fractionation of intestinal mucosa was complicated by the mucus released from the goblet cells on homogenization. This difficulty was overcome by using a fractionation method similar to that used by Glover & Green (1957). Mucosa from rat small intestine was homogenized with 20 vol. of 0·25M-sucrose in a Potter–Elvehjem homogenizer, and the homogenate centrifuged at 3 600 000*g*.min. The supernatant was decanted and the residue homogenized in 40 vol. of 0·25M-sucrose. The homogenate was centrifuged at 10 000*g*.min. and the cloudy supernatant decanted. The gelatinous residue was subjected twice more to this procedure. The final residue was taken as the mucus fraction. The combined supernatants from the 10 000*g*.min. sedimentations were centrifuged at 148 000*g*.min. to sediment mitochondria and at 3 600 000*g*.min. to sediment microsomes. The microsomal supernatant was combined with the initial supernatant as the final supernatant fraction.

All the fractions were assayed for enzymes hydrolysing L-leucylglycine and glycylglycine. The assay method was that used by Robinson & Shaw (1960). The hydrolysis of L-Leu–Gly was measured at pH 8·3 and the hydrolysis of Gly–Gly was measured at both pH optima, pH 7·5 and pH 8·0 (Robinson & Shaw, 1960) in the presence of mM-Co^{2+}.

The supernatant contained 90% of the Leu.Gly hydrolysing activity, with the remaining 10% residing mainly in the microsome fraction. The overall recovery was 94%. The enzymes hydrolysing Gly.Gly were similarly distributed. At both pH 7·5 and pH 8·0, 84–85% of the activity was in the supernatant fraction. The microsomes contained more of the remaining activity than the mitochondria. The overall recoveries at pH 7·5 and pH 8·0 were 92 and 104% respectively.

Approximately 64% of the protein was in the supernatant fraction with 10%, 6% and 18% in the microsomal, mitochondrial and mucus fractions.

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The Effect of Oligomycin on Cation Transport in Slices of Rat Liver

By G. D. V. van Rossum.† (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

The use of oligomycin as an inhibitor of oxidative phosphorylation (Lardy, Johnson & McMurray, 1958) has made it possible to investigate whether energy-rich intermediates of oxidative

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phosphorylation other than ATP can provide the energy necessary for active cation transport.

Slices of rat liver lost potassium and gained sodium when preincubated at 1°. The reversal of these cation movements during subsequent incubation at 38° was taken as a measure of active transport. The rate of respiration of the slices was determined during the incubation at 38°.

Oligomycin inhibited the active transport of sodium and potassium in slices of adult rat liver by a maximum of 50 % and respiration by only 20 %. Cation transport and respiration are both inhibited 90 % by cyanide. Thus, the oligomycin-resistant transport derived its energy largely from the oligomycin-resistant respiration, while the oligomycin-sensitive transport was dependent upon the oligomycin-sensitive respiration. The respiratory inhibition by oligomycin was largely released by dinitrophenol suggesting that, as in isolated mitochondria, the oligomycin-sensitive respiration is coupled to the formation of intermediates of oxidative phosphorylation beyond the point of action of dinitrophenol.

Liver slices prepared from late foetal rats can obtain energy for about half their cation transport from anaerobic glycolysis (van Rossum, 1961). Oligomycin was found to inhibit this glycolysis-dependent transport by 50 %. The rate of glycolysis was not inhibited.

Representing oxidative phosphorylation as

$$\text{Respiration} \rightarrow \sim_1 \rightleftharpoons \sim_2 \rightleftharpoons \sim P \rightleftharpoons ATP,$$

oligomycin acts in the region of the $\sim_2$ compound (Huijing & Slater, 1961). The effects of oligomycin on cation transport may be explained if the energy for part of the transport is provided by $\sim_1$ (cf. Slater, Tager & Snoswell, 1962) and for the rest by $\sim P$ or ATP. In the foetal liver the ATP produced by anaerobic glycolysis could allow the synthesis of $\sim_1$ by reversal of the oxidative phosphorylation mechanism (Slater, 1953). Oligomycin would inhibit this process and so inhibit the cation transport which depends on $\sim_1$, but not that depending on $\sim P$ or ATP. Conversely, oligomycin would inhibit only that part of the aerobic cation transport of the adult liver which depends on $\sim P$ or ATP.

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Lipid Metabolism of Rat Thymocytes and the Transfer of Lipid from Thymocytes to Plasma

By C. E. Rowe.† (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Studies are reported on the incorporation of [1-^{14}C]acetate and [^{32}P]orthophosphate into the lipids of rat-thymocyte suspensions in Krebs–Ringer bicarbonate and in plasma. A smear, stained with methyl green and pyronin, from a typical preparation contained 96·6 % small lymphocytes (thymocytes), 2·8 % large lymphocytes and 0·6 % other cells including polymorphs and red cells. Thymocytes differed from the residual thymus tissue from which they were initially separated in respect to (i) phospholipid composition, (ii) the incorporation of [^{32}P]orthophosphate into the different phospholipid classes, (iii) the incorporation of [^{14}C]acetate into unsaponifiable lipid, neutral saponifiable lipid, and phospholipid.

When thymocytes were incubated in plasma with [^{14}C]acetate there was rapid incorporation into the lipids of cells and plasma, indicating that the rate of transfer of lipid from cells to plasma was fast compared with biosynthesis. A detailed analysis was performed of the lipids of cells and plasma obtained after the incubation of thymocytes ($2{\cdot}7 \times 10^9$) in heparinized plasma (7·0 ml.), with 40 μc of [1-^{14}C]acetate and 1340 μc of [^{32}P]-phosphate at 37° for 4 hr.

The phospholipids were analysed by chromatography on a column of silica gel with the solvent chloroform–methanol (Hanahan, Dittmer & Warashina, 1957). Successive eluate fractions were grouped according to their ^{32}P-radioactivities and their phosphorus content. The most abundant thymocyte phospholipid was lecithin (59 %). Phosphatidyl ethanolamine and phosphatidyl serine together accounted for a further 32 % of the total phospholipid phosphorus. Lecithin contained 55 % and 57 % of the total phospholipid ^{32}P and ^{14}C radioactivities respectively. Three lecithin fractions contained the same ratios of ^{32}P to ^{14}C.

Radioactive phospholipids were found in the plasma. The ratios of ^{32}P to ^{14}C in the various phospholipid classes were different from those of the same classes from the cells. There was preferential transfer to the plasma of a phospholipid corresponding to 4 % of the total cellular phospholipid phosphorus. This component on a silica column in chloroform–methanol, and on silica impregnated paper in di-isobutyl ketone–acetic acid–water (40:30:7, v/v; Marinetti & Stotz, 1956) ran in positions expected for inositol phosphatide.

The neutral lipids of both thymocytes and plasma were likewise analysed by chromatography on a

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column of silica gel with diethyl ether–light petroleum (b.p. 40–60°) mixtures (Hirsch & Ahrens, 1958).

Of the total lipid synthesized from [^{14}C]acetate, 79% of the cholesterol, and 62% of the component with the chromatographic behaviour of inositol phosphatide, were transferred to the plasma. Only 2% of the lecithin synthesized from acetate was transferred.

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Levels of Oxidized and Reduced Pyridine Nucleotides in Rat Retina

By T. F. Slater,* H. Heath and C. N. Graymore. (*Department of Chemical Pathology, University College Hospital Medical School and Department of Pathology, Institute of Ophthalmology, London*)

The metabolism of the retina is characterized by a very high rate of glucose utilization, and both the Embden–Meyerhof pathway and the pentose phosphate route have been shown to operate *in vitro* (Cohen & Noell, 1960). Changes in the relative magnitudes of these two pathways have been shown to occur under various pathological conditions in other tissues and may be reflected in alterations in the levels of the oxidized and reduced pyridine nucleotides. Owing to the possible relationship of these changes to the development of pathological lesions in the eye, it was thought desirable to determine the levels of these nucleotides in the retina. This has entailed the development of an ultramicro-modification of the method of Slater & Sawyer (1962) by which as little as 2×10^{-11} mole of nucleotide can be measured; 10 mg. wet wt. of tissue is adequate for the determination of either the oxidized or reduced forms of the two nucleotides. Mean values ± S.E.M. found for NAD, $NADH_2$, NADP, $NADPH_2$ in the retina of the normal adult male albino rat were respectively: 226 ± 13 (14), 46 ± 3·0 (18), 8·7 ± 0·9 (13), 25·0 ± 3·0 (14); these values are in μg./g. wet wt. of retina and the numbers of determinations are shown in parentheses. These levels are similar to the values found for brain by Lowry, Passonneau, Schulz & Rock (1961) but differ markedly from values found for liver (Slater & Sawyer, 1962). The ratios NAD:$NADH_2$ and $NADPH_2$:NADP in retina were 5·0 ± 0·4, and 2·5 ± 0·2. The relatively low $NADPH_2$:NADP ratio contrasts with the high ratio found in other tissues known to have an active pentose phosphate pathway (cf. Dumont, 1960) and this supports the view that although the pentose shunt can occur in adult retina it does not appear to be the major route (Cohen & Noell, 1960). Details of the method and its application to the retina in various conditions will be described and will be published in detail later.

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Relationships between Ribonucleic Acid and Protein of Some Plant Viruses

By R. H. Symons,* M. W. Rees and R. Markham. (*Agricultural Research Council, Virus Research Unit, Huntingdon Road, Cambridge*)

Six strains of turnip yellow mosaic virus (TYMV) have been analysed for the amino acid composition of the proteins, the base ratios and the pancreatic-ribonuclease digestion products of the RNA. The results indicate that the TYMV strains fall into two distinct groups. Group I has an average base ratio (molar %) of

$$G/A/C/U = 17.3/22.5/38.3/21.9$$

with no significant differences between the three strains. The values for group II are

$$G/A/C/U = 16.6/21.4/41.6/20.3$$

with one strain differing slightly from the other two. The quantitative estimation of the mono-, di- and most of the tri-nucleotides resulting from ribonuclease digestion (Staehelin, Peterson & Sober, 1959; Staehelin, 1961) has shown a number of significant differences between all strains but there remains the marked tendency for the strains to fall into two groups.

The amino acid compositions of the proteins within each group are similar but there are major differences between the two groups. The values of residues of some amino acids, calculated per protein sub-unit (mol.wt. 20 000), emphasize these differences. Group I: Lys 7, His 3, Arg 3, Asp 11, Thr 26, Phe 5; Group II: Lys 4, His 5, Arg 6, Asp 18, Thr 20, Phe 3.

The protein sub-unit contains 189 residues of amino acids; if a single base triplet can code each amino acid, only one-twelfth of the nucleic acid

found (mol.wt. 2×10^6) would be necessary. This implies that only a part of the viral RNA may be necessary to code virus protein. Furthermore, the various codes recently proposed (Martin, Matthaei, Jones & Nirenberg, 1962; Speyer, Lengyel, Basilio & Ochoa, 1962; Wittmann, 1961) all demand a preponderance of uracil and the results obtained with TYMV nucleic acid are far too low in uracil to permit matching with these codes. It is interesting that the base ratio for coding RNA calculated from these codes and the amino acid composition of whole plant proteins is little different from that calculated for TYMV; in contrast the amino acid compositions of the respective proteins are quite different.

Further work on RNA–protein relationships in other plant viruses is in progress.

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The Metabolism of [^{14}C]Methylcyclohexane

By C. C. Tao and T. H. Elliott. (*Department of Pharmaceutics, The University of Singapore*)

Bernhard (1937) was unable to find unchanged methylcyclohexane or any of its metabolites in the urine of dogs. Treon, Crutchfield & Kitzmiller (1943) found, in the rabbit, only 4–5% of the administered dose to be excreted as a conjugated glucuronide. Williams (1959) states that it has been suggested that the primary reaction in the metabolism of methylcyclohexane is ring fission as a preliminary to complete oxidation. Studies of the metabolism of [^{14}C]methylcyclohexane with the methods of Elliott, Parke & Williams (1959) show that ring fission is a minor metabolic route (approximately 5%) in rabbits receiving doses of 2–2·5 m-moles/kg. Some is excreted unchanged in the breath (10%). The majority of the administered dose (65%) is excreted in the urine conjugated with glucuronic acid. The remainder is retained in the tissues. The percentages of the several metabolites were *cis*-2-methylcyclohexanol 0·6%, *trans*-2-methylcyclohexanol 1·2%, *cis*-3-methylcyclohexanol 11·5%, *trans*-3-methylcyclohexanol 10·5%, *cis*-4-methylcyclohexanol 2·4%, *trans*-4-methylcyclohexanol 14·7%. Ketones were not detected. The methyl group was oxidized to some extent, 0·3% of cyclohexylmethanol being detected together with 1·9% total benzoic acid (part of the latter, however, was found to be an artifact).

In the related field of steroid metabolism the consensus of opinion (Hayano, Gut, Dorfman, Sebek & Peterson, 1958; Bergstrom, Lindstet, Samuelson, Corey & Gregoriou, 1958; Corey, Gregoriou & Peterson, 1958; Grant, 1956) is that hydroxylation of steroids occurs as a result of a direct replacement of ring hydrogen by a hydroxyl group, but whether the reaction involves electrophilic or free-radical substitution has not been settled, and the nature of the enzyme system is unknown.

The distribution pattern of hydroxylation in methylcyclohexane offers some support on chemical grounds to the hypothesis that the hydroxylation is affected by a free-radical reaction, the varying proportions found being controlled by steric factors. If electrophilic substitution had been the mechanism, 2-methylcyclohexanols would have been expected to be the major metabolites because the C-1 position in methylcyclohexane, having the highest electron density due to the inductive effect of the methyl group is the site most liable to electrophilic attack.

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The Stereospecific Metabolism of Methylcyclohexane Derivatives

By C. C. Tao and T. H. Elliott. (*Department of Pharmaceutics, The University of Singapore*)

Rabbits metabolize the isomeric methylcyclohexanols and methylcyclohexanones to the corresponding thermodynamically more stable alcohols. Thus racemic *trans*-2-, *cis*-2-methylcyclohexanol, and 2-methylcyclohexanone all yield *trans*-2-

methylcyclohexanol which is racemic when the *trans*-alcohol is fed but *dextro*-rotatory when the *cis*-alcohol or the ketone is fed. Racemic *cis*- and *trans*-3-methylcyclohexanols and 3-methylcyclohexanone yield racemic *cis*-3-methylcyclohexanol. *Cis*- and *trans*-4-methylcyclohexanols and 4-methylcyclohexanone yield *trans*-4-methylcyclohexanol.

These stereochemical metabolic differences are consistently explicable in terms of the conformations of the substrates and Vennesland's (1958) hypothesis of the role of NADH in dehydrogenation reactions. 2-, 3- and 4-Methylcyclohexanones are reduced by NADH and horse-liver alcohol dehydrogenase. The K_m values are $6{\cdot}7 \times 10^{-2}$M, 5×10^{-3}M, and $1{\cdot}7 \times 10^{-3}$M, respectively.

A face-to-face contact is visualized between the coenzyme and substrate permitting only one of the stereospecific hydrogens on the C-4 atom of NADH to attack the carbonyl carbon atom of the substrate ketone, ordinarily, from the most hindered side thus producing an equatorially substituted alcohol. Further, only one face of the coenzyme can be utilized by any given substrate, which is consequently subject either to an α- or β-specific attack. Examination of Courtauld models of the various substrates, with the absolute configurations recently established (Djerassi & Krakower, 1959; Djerassi, Beard, Elliott & Tao, 1962), reveals that with some of the NADH complexes there are steric interactions between the $CONH_2$ group of the coenzyme and the alkyl substituent of the substrate (particularly with the (−)-2-ketone) of sufficient magnitude to prevent effective hydrogen transfer. Hence in a racemic mixture of the 2-ketone essentially the (+)-isomer will be attacked, giving an optically active metabolite. Where this steric interaction does not occur, e.g. with both forms of 3-methylcyclohexanone, hydrogen transfer to either conformer occurs with equal facility giving a racemic metabolite.

The inversion of the alcohols and the optical activity of the metabolites is explained similarly. Equatorial hydroxyl groups are conjugated directly, but axially substituted alcohols (*cis*-2-, *trans*-3- and *cis*-4-methylcyclohexanols) first react with NAD^+ to yield a ketone, by using one specific face of the coenzyme, and then with NADH by using the other specific face to yield the inverted alcohol, which for the same reasons as were adduced for the ketones is optically active only in the case of the *trans*-2-alcohol.

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Interplay Between Cell Sap and Subcellular Particles as Revealed by Thyroxine-Binding

By J. R. Tata,*† L. Ernster and Eva M. Suranyi. (*The Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden*)

Studies on the mechanism of action of biologically active substances are often based on a direct interaction between cellular constituents and the active compound. Our studies on the action of thyroid hormones at the cell level (Tata, Ernster & Lindberg, 1962) prompted a detailed investigation of factors governing the interaction between the major subcellular fractions and L-thyroxine and its derivatives. Isolated rat-liver and skeletal-muscle mitochondria, microsomes and their membraneous subfractions exhibit an intense thyroxine-binding which is in some ways similar to that of liver mitochondria described by other workers (Klemperer, 1955; Tapley & Basso, 1959; Tonoue & Matsumoto, 1961). This interaction with subcellular particles, mainly due to electrostatic forces, is, however, quite different from thyroxine-binding by serum proteins (see Tata, 1962) and appears to be linked to the lipid-rich structural components.

In contrast with the strong thyroxine-binding exhibited by isolated particles *in vitro*, endogenous ^{131}I-labelled thyroid hormone (or tracer amounts of radioactive L-thyroxine added to rat-liver and muscle homogenates) was not significantly concentrated in the particulate fractions; the largest fraction was recovered in the cell sap (microsome-free supernatant). The presence of cell sap in the suspension or washing media reduced thyroxine-binding *in vitro* by previously washed mitochondria and microsomes. The inhibitory effect of the cell sap was due to two factors: (1) Competition by thyroxine-binding sites in the cell sap (Tata, 1958; Kallee, 1960). (2) A modification of the thyroxine-binding properties of the particles by the cell sap. This effect was particularly marked for liver mitochondria. The implications of the competitive and modifying actions of cell sap on thyroxine-binding by the particulate cellular constituents will be discussed with reference to the biological activity of thyroid hormones (see Tata, 1962).

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Mitochondrial Ribonucleic Acid and its Role in the Incorporation of Amino Acids into Mitochondrial Protein

By D. E. S. TRUMAN* and A. KORNER. (*Department of Biochemistry, University of Cambridge*)

Well-washed rat-liver mitochondria contain about 1·4 mg. of RNA/100 mg. of mitochondrial protein (Truman & Korner, 1962). This RNA was extracted with lauryl sulphate and phenol and its sedimentation coefficient (S) was determined in the analytical ultracentrifuge. Only one peak was found with an S value of 8·39s which is intermediate between that of soluble RNA and that of ribosomal RNA. The presence of an RNA with this sedimentation coefficient indicates that the mitochondria are free from microsomal contamination and lends support to the contention that the amino acid incorporation into proteins of isolated rat-liver mitochondria is not caused by contaminating microsomes (Roodyn, Reis & Work, 1961; Truman & Korner, 1962).

RNA was also found in two subfractions of well-washed mitochondria. One was obtained by treating mitochondria with sodium deoxycholate when particles with sedimentation coefficients of 77s together with smaller components of 45s and 120s were obtained and partially separated on a sucrose gradient. These particles contain about 15mg. of RNA/100mg. of protein and they appear to be associated with that portion of the mitochondrial protein which, we have found, is most rapidly labelled after injection *in vivo* of radioactive amino acids. The second submitochondrial fraction was prepared by disrupting mitochondria when a soluble extract was obtained which could replace the cell sap in the ribosomal incorporating system of Korner (1961). The extract was still active after precipitation at pH 5 and redissolving at pH 7·2 and the addition of GTP caused marked stimulation of incorporation into the ribosomes. This mitochondrial extract contains RNA but no appreciable incorporation of amino acids into the RNA was detected when the extract was incubated with [^{14}C]leucine and ATP.

The results support the idea that amino acid incorporation into mitochondrial protein resembles in general that of the ribosomal system. The presence of a new type of RNA in mitochondria which is not present in the cell sap or in the ribosomes may indicate that the precise function of the RNA in this system differs from that in the ribosome system.

We thank the Medical Research Council for a research grant and for a scholarship to one of us (D.E.S.T.).

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Development of Respiration in Anaerobically Grown Yeast

By E. R. TUSTANOFF*† and W. BARTLEY. (*Department of Biochemistry, University of Oxford*)

The study of the so-called 'oxygen adaptation' in anaerobic yeast has been extensively investigated by Slonimski (1953, 1956). It was the purpose of this present investigation to obtain further information about this phenomenon.

Saccharomyces cerivisiae was grown under N_2 at 30° in a liquid medium consisting of glucose, hydrolysed casein, yeast extract, sodium lactate, inorganic salts, Tween 80, wheat-germ oil and ergosterol, and was harvested towards the end of the log phase. The onset of respiration was tested by incubating the washed cells (3 mg. dry wt.) in air in a medium of glucose (33 mM) and phosphate (66 mM), pH 5·8. When supplemented with hydrolysed casein (7 mg./ml.), respiration commenced abruptly at about 2 hr. and rapidly increased to a maximum ($Q_{O_2} = 30-90$, depending upon experimental conditions) and thereafter fell rapidly to a Q_{O_2} between 20 and 40; whereas in the absence of casein the increase in respiration was gradual. The magnitude of the maximum Q_{O_2} obtained depended on the glucose concentration in the growth medium (5·4%: $Q_{O_2} \cong 30$; 0·7%: $Q_{O_2} \cong 90$) and the physiological age of the harvested cells. Cells harvested at the onset of the stationary phase had a lower respiratory peak and those from the stationary phase had no peak (max. $Q_{O_2} \cong 12$). The time of the onset of the respiratory peak was influenced by varying the amount of yeast in the adapting medium. With 7·2, 5·4 and 3·6 mg. dry weight the respiratory peak occurred at 2, 3 and 4 hr. respectively, but with 1·8 mg. no peak appeared. With 3 mg. dry wt. of yeast, the time of development of respiration with 100 and 200 μmoles of glucose was 2·5 and 4·5 hr. but with 300 or 400 μ-

† Fellow of the Life Insurance Medical Research Fund.

moles there was little or no respiration. This suggested that the presence of glucose was inhibiting the formation of respiratory enzymes. Analysis of the incubation medium showed that respiration did not occur until the glucose had fallen to a very low level. Ethanol concentration was maximal at the peak of respiration and did not change thereafter. Fermentation ceased when maximum respiration occurred. Cells when incubated anaerobically until CO_2 evolution ceased and then transferred to aerobic conditions respired substantially and without a lag period.

In contrast with glucose-grown cells, anaerobic galactose-grown cells of the same physiological age respire immediately. These experiments suggest that glucose concentration is more effective in repressing the formation of respiratory enzymes than anaerobiosis under these experimental conditions.

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Vitamin B_{12} Deficiency and the Excretion of Methylmalonic Acid by the Human

By A. M. White.* (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Dimethylbenzimidazolylcobamide coenzyme (Weissbach, Toohey & Barker, 1959) is required for the isomerization of methylmalonyl-coenzyme A to succinyl-coenzyme A by cell-free extracts from *Propionibacterium shermanii* (Stadtman, Overath, Eggerer & Lynen, 1960) and rat liver (Gurnani, Mistry & Johnson, 1960). This isomerization is blocked also in the protozoan *Ochromonas malhamensis* when the organism is in a state of vitamin B_{12} deficiency (Arnstein & White, 1962). Although the coenzyme form of vitamin B_{12} occurs in human liver (Toohey & Barker, 1961), the existence of methylmalonic acid as a human metabolite has not so far been demonstrated. It has now been shown that methylmalonic acid occurs in normal human urine and the amount excreted is greatly increased in patients suffering from megaloblastic anaemia due to vitamin B_{12} deficiency.

Sodium [2-^{14}C]methylmalonate of known specific radioactivity, prepared by incubating vitamin B_{12}-deficient *Ochromonas malhamensis* with sodium [2-^{14}C]propionate (Arnstein & White, 1962), is added to a portion (usually 200 ml.) of a 24 hr. urine specimen. After bringing the urine to pH 3 with sulphuric acid, continuous ether extraction is carried out overnight, and the ethereal extract, after removal of the solvent, is applied to a column (1·5 cm. × 42 cm.) of Dowex 1 anion-exchange resin in the acetate form. Acids weaker than methylmalonic acid, including succinic acid, are eluted with acetic acid (700 ml., 0·5N; 700 ml., N). The remaining ether-soluble acids are eluted with N-HCl (300 ml.). Pure methylmalonic acid is isolated from this eluate and purified to constant specific radioactivity by using a Celite column (Swim & Krampitz, 1954). From the dilution in specific radioactivity which has occurred, the amount of methylmalonic acid in the original urine sample is calculated. The purity of the isolated methylmalonic acid was checked, in addition, by gas chromatography.

The normal excretion of methylmalonic acid was found to be 4–5 mg./24 hr. and in cases of megaloblastic anaemia due to vitamin B_{12}-deficiency increases were observed (50–90 mg. in 24 hr.). Upon administration of vitamin B_{12} the amount excreted returns to normal.

Thus, in the human, vitamin B_{12} appears to be involved in the catabolism of methylmalonic acid.

It is suggested that accumulation of methylmalonic acid in the tissues may be toxic and give rise to some or all of the symptoms of pernicious anaemia.

Thanks are expressed to Dr E. V. Cox for the 24 hr. urine samples from pernicious-anaemia patients.

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Substrate Specificity of Microbial and Plant Dihydroxy Acid Dehydratases

By R. L. Wixom, M. Kanamori and J. W. Blankenship (introduced by D. D. Woods). (*Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock, U.S.A.*)

The enzymic dehydration of $\alpha\beta$-dihydroxy acids, a step in valine and isoleucine biosynthesis, has been briefly studied with regard to specificity (Wixom, Blankenship & Kanamori, 1961). This work has now been extended to three other microbial extracts (*Pseudomonas aeruginosa, Serratia*

marcescens, and *Proteus morganii*) and two more plant sources (*Chlorella pyrenoidosa* and *Triticum vulgare*). Each extract catalysed keto acid formation from DL-$\alpha\beta$-dihydroxyisovalerate at a faster rate than from the homologous DL-$\alpha\beta$-dihydroxy-β-methylvalerate. The *threo*-isomer of DL-$\alpha\beta$-dihydroxybutyrate was dehydrated more rapidly than the *erythro*-isomer by these five extracts. Except for *S. marcescens*, keto acids were not formed from DL-$\alpha\beta$-dihydroxy-β-phenylpropionate, DL-*threo*- and *erythro*-$\alpha\beta$-dihydroxynonanoate.

The crude extracts were examined for other dehydratases, which, if present, might also dehydrate the dihydroxy acids. The contaminating activities found were serine dehydratase of *P. aeruginosa*, and serine dehydratase and homocysteine desulphydrase of *P. morganii*. These three activities were inhibited by 0·02M-hydrazine or semicarbazide, which had no effect on the dihydroxy acid dehydratase. Extracts of *Escherichia coli* contained dihydroxy acid-, serine- and threonine-dehydratase activities, but their ratios varied widely under four different growth conditions. This evidence distinguishes between the various microbial dehydratases.

The substrate specificity of spinach dihydroxy acid dehydratase was also examined during its 120-fold purification (Wixom & Kanamori, 1962). With 0·02M of the respective substrates, the ratios of the rates of formation of product (α-oxoisovalerate:α-oxo-β-methylvalerate and α-oxoisovalerate:α-oxobutyrate) were relatively constant at each of five purification steps. The first ratio had the same magnitude at six different pH values, similar to the purified *E. coli* dehydratase (Myers, 1961). Hence the dehydration of the four acids is intrinsic to the dehydratase. The observed similar substrate specificity is consistent with the extension of the known parallel pathway of valine and isoleucine biosynthesis in *E. coli*, *Neurospora crassa* and *Saccharomyces cerevisiae* to the above new biological systems.

This investigation was supported by grant RG-6626 of the National Institutes of Health, U.S.P.H.S.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **417th Meeting** *of the Society was held in the Department of Biochemistry, University of Edinburgh, on Friday,* 8 *June* 1962, *when the following communications were presented:*

COMMUNICATIONS

Azurin, a Blue Bacterial Protein

By I. W. Sutherland and J. F. Wilkinson. (*Department of Bacteriology, University of Edinburgh*)

During chromatography and electrophoresis of fractions from *Bordetella pertussis* cells, the presence of a blue protein was reported (Hammarsten, Palmstierna & Meyer, 1959). This blue protein has now been extracted from several strains of *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* and has been called 'azurin'. In crude extracts, azurin is associated with a bacterial cytochrome of the *c* type. The blue protein has been purified by chromatography on ion-exchange cellulose and dextran materials. The product was homogeneous when examined by electrophoresis and by ultracentrifugation.

Azurin is a water-soluble protein of low molecular weight, about 14 600, and it appears to contain one atom of copper per protein molecule. Continuous spectra on all preparations show absorption maxima at 279 mμ and 625 mμ in the oxidized state. The latter peak disappears on reduction, which can be achieved with dithionite, cysteine, glutathione and ascorbic acid, and also by cell-free bacterial extracts in the presence of succinate. The reduction of azurin is reversible and oxidation occurs in air, the process being accelerated by ultrasonic extracts of *Bordetella* cells. Potentiometric titration indicated an oxidation–reduction value of +395 mv at 20°.

In an attempt to elucidate the function of azurin, a wide range of Gram-negative bacterial species were examined for the presence of this or similar proteins. From three species, *Alcaligines faecelis*, *A. denitrificans* and *Pseudomonas fluorescens*, a blue protein was isolated by the same methods as were used for azurin. These materials resembled azurin in many of their physical and chemical properties. Another blue protein has been reported in *Pseudomonas aeruginosa* (Horio, 1958*a*, *b*). Although this resembled azurin in many respects, it could not be oxidized with molecular oxygen. No detectable differences in the amino acid composition of the various bacterial blue proteins could be observed from two-dimensional paper chromatography of acid hydrolysates.

The authors wish to thank Dr H. J. Cruft for the analytical ultracentrifuge and electrophoresis studies.

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Conversion of Glutamic Acid into Aspartic Acid in Cerebral Cortex Slices

By M. M. Cohen, G. R. Simon, J. F. Berry and E. B. Chain. (*Division of Neurology, University of Minnesota Medical School, Minneapolis, U.S.A.*)

Glutamic acid is unique among amino acids in its ability to support respiration of cerebral tissue *in vitro*; however, its pathway of oxidation remains to be delineated. The conversion of glutamate into aspartate by various tissue preparations has been demonstrated and assumed to proceed through transaminations involving tricarboxylic acid cycle intermediates (Krebs & Bellamy, 1960). In our previous studies employing cerebral cortex slices glutamate, when utilized as the sole added substrate, gave rise to 40% of the carbon dioxide produced. Addition of glucose increased oxygen uptake and carbon dioxide production, but 40% of the carbon dioxide produced still derived from glutamate (Chain, Cohen & Pocchiari, 1962). If glucose and glutamate shared portions of a common oxidative pathway, the proportion of carbon dioxide derived from glutamate should have decreased. These data suggested that glutamate was principally oxidized through a pathway not involving the tricarboxylic acid cycle. This hypothesis was tested by incubation of guinea-pig cerebral-cortex slices in media containing [^{14}C]glutamic acid followed by isolation of keto acids and amino acids employing Celite 535 and Dowex 50 columns respectively. Results indicate aspartate formation from glutamate without major contribution by transamination to α-oxoglutarate or reentry into the tricarboxylic acid cycle through conversion into succinate.

When [U-^{14}C]glutamate was employed as substrate, the relative specific activity (R.S.A.) of aspartate approximated 1 ($0{\cdot}92 \pm 0{\cdot}06$) (R.S.A. for each compound studied = counts per min. per C atom of compound/counts per min. per C atom of glutamate). This indicates aspartate formation without transamination or other reaction involving tricarboxylic acid cycle intermediates, since admixture with these compounds would result in considerably lower values. The R.S.A. of γ-aminobutyrate was 0·2 and the R.S.A.s of pyruvate, fumarate, α-oxoglutarate, citrate and succinate were between 0·1 and 0·19.

Additional evidence that glutamate is converted into aspartate in cerebral slices through mechanisms outside the tricarboxylic acid cycle was obtained by incubation with [1-^{14}C]- or [5-^{14}C]-glutamate. With [1-^{14}C]glutamate the R.S.A. of aspartate was $0{\cdot}78 \pm 0{\cdot}02$ indicating formation principally through decarboxylation of unlabelled carbon atoms. When [5-^{14}C]glutamate was employed, the R.S.A. of aspartate was $0{\cdot}22 \pm 0{\cdot}02$. These data indicate aspartate formation from glutamate principally through a decarboxylation of $C_{(5)}$ not involving tricarboxylic acid intermediates or conversion into γ-aminobutyrate.

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Deoxyribonuclease and Deoxyribonucleic Acid Nucleotidyltransferase of Landschutz Ascites-Tumour Cells

By H. M. Keir and Georgina L. Aird. (*Biochemistry Department, The University of Glasgow*)

Enzymic synthesis of polydeoxyribonucleotide in soluble extracts of mammalian cells is stimulated by limited pretreatment of the primer with pancreatic deoxyribonuclease (Sarkar, 1961; Keir, Binnie & Smellie, 1962) or by inclusion of that enzyme in the reaction mixture during synthesis (Mantsavinos & Canellakis, 1959). These reports suggested that the terminal groups of the primer influenced the DNA nucleotidyltransferase activity. We have therefore investigated the deoxyribonucleases active at pH 7 and pH 5, deoxyribonucleases I and II respectively (Cunningham & Laskowski, 1953), in extracts of ascites-tumour cells. Both activities were located predominantly in the particulate fractions of the cell, the specific activity of deoxyribonuclease I being similar in the nuclear and mitochondrial–microsomal fractions, while that of deoxyribonuclease II was two times higher in the nuclei than in the mitochondria-microsomes. Although soluble extracts prepared quickly from osmotically disrupted cells contained little deoxyribonuclease, increased yields of the enzymes were obtained when KCl and potassium phosphate buffer, pH 7·5, were added immediately after disruption and shortly before centrifuging.

Examination of deoxyribonuclease I and DNA nucleotidyltransferase activities under comparable conditions showed that extracts prepared without delay in KCl–phosphate buffer contained three times as much deoxyribonuclease as soluble extracts prepared before addition of KCl–phosphate buffer, that thermally denatured DNA was a more effective substrate than native DNA, and that the increase in deoxyribonuclease was accompanied by increases in total and specific activities of DNA nucleotidyltransferase. Soluble extracts prepared from disrupted cells held for 3 hr. in the presence or absence of KCl–phosphate buffer showed, respectively, 15 and 35% less DNA nucleotidyltransferase activity than corresponding extracts prepared without delay. Acid fractionation of these four types of soluble extract effected three- to six-fold purifications of DNA nucleotidyltransferase, removed much of the deoxyribonuclease I and, in yielding similar total and specific activities for the transferase, revealed that prolonged exposure of the disrupted cells to KCl–phosphate buffer had extracted from the particles an inhibitory factor which was not deoxyribonuclease I.

We attribute the initial lag in enzymic synthesis of polydeoxyribonucleotide to a relative deficiency of 3′-hydroxyl groups in the primer, and conclude that the lag is overcome by the action of deoxyribonuclease I in the DNA nucleotidyltransferase preparations as incubation proceeds.

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Activation and Inhibition of Deoxyribonucleic Acid Nucleotidyltransferase from Ascites-Tumour Cells

By H. M. Keir. (*Biochemistry Department, The University of Glasgow*)

Limited pretreatment of primer DNA with pancreatic deoxyribonuclease brings about a 60%

increase in its ability to prime the DNA nucleotidyltransferase system from Landschutz ascites-tumour cells (Keir, Binnie & Smellie, 1962). Further investigation of this phenomenon has involved digestion of DNA by pancreatic deoxyribonuclease followed by separation of the oligonucleotide products by ion-exchange chromatography on columns of diethylaminoethyl-Sephadex. The products, which are of the 5′-phosphoryl- and 3′-hydroxy-terminal variety (Laskowski, 1961), contain fractions which, when included in the standard assay system for DNA nucleotidyltransferase, increase the rate of synthesis of polydeoxyribonucleotide. Similar procedures employing purified splenic deoxyribonuclease (Koerner & Sinsheimer, 1957) provided oligonucleotide fractions bearing 5′-hydroxy- and 3′-phosphoryl-terminal groups. These fractions reduced the rate of synthesis of polydeoxyribonucleotide in the DNA nucleotidyltransferase system. Removal of terminal phosphoryl groups from 5′-phosphoryl-terminal oligonucleotide material (average chain length = 4·4 nucleotidyl units) by bacterial alkaline phosphatase yielded oligonucleotide which still stimulated the reaction but at a reduced level. In contrast, removal of the terminal phosphoryl groups from a 3′-phosphoryl-terminal oligonucleotide fraction (average chain length = 6·1 nucleotidyl units) abolished the inhibitory effects and produced oligonucleotide which stimulated the reaction rate. The stimulatory activities of the dephosphorylated oligonucleotides so derived from 3′-phosphoryl- and 5′-phosphoryl-terminal fractions were of a similar order. Treatment of thermally denatured DNA with bacterial alkaline phosphatase during the DNA nucleotidyltransferase assay resulted in an increase in initial reaction rate but gave a decrease in overall synthesis. These observations are interpreted to be the consequence of the removal of 3′- and 5′-phosphoryl-terminal groups, respectively, from the DNA.

Dialysis of soluble extracts of Landschutz ascites-tumour cells resulted in a lowering of the capacity of the extracts to synthesize polydeoxyribonucleotide. The synthetic effect was restored to normal by addition of boiled soluble extract to the assay system. Preliminary observations indicate that the stimulatory factor in the boiled extract is not of low molecular weight.

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The Formation of Thymidine-5′-Triphosphate by Enzymes from Landschutz Ascites-Tumour Cells

By H. J. Grav and R. M. S. Smellie. (*Department of Biochemistry, The University of Glasgow*)

Two mechanisms have been postulated for reactions leading to the formation of thymidine 5′-triphosphate (TTP) from thymidine (TdR) (Weissman, Smellie & Paul, 1960; Bianchi, Butler, Crathorn & Shooter, 1961): (*a*) Stepwise addition of orthophosphate to TdR to yield successively thymidine 5′-monophosphate (TMP), thymidine 5′-diphosphate (TDP) and TTP. (*b*) Phosphorylation of TdR to yield TMP followed by the addition of pyrophosphate to form TTP.

The mechanism of formation of TTP by enzymes from Landschutz ascites-tumour cells has been examined using an improved method for the complete separation of the thymidine nucleotides on columns of ECTEOLA-cellulose.

Studies on the time course of formation of TTP from [^{3}H]TdR have invariably shown that labelled TMP and TDP may be detected very soon after the start of the incubation. On no occasion was labelled TTP detected earlier than labelled TDP. As the incubation proceeds, [^{3}H]TTP accumulates so that after incubation for 1 hr. as much as 60 % of the total radioactivity is located in TTP. The proportion of the total radioactivity located in TDP never exceeds 20 %.

In experiments in which TDP labelled in the α-phosphate with ^{32}P was used as substrate it was shown that the diphosphate was rapidly and extensively converted into TTP, more than 60 % of the total radioactivity being recovered in TTP. Moreover, the addition of unlabelled TMP to the reaction mixture resulted in only a very slight reduction in the proportion of the total counts recovered as TTP.

When the reaction was followed using [^{32}P]TMP as substrate in the presence of added non-labelled TDP and TTP it was found that the specific activity of the TDP started to rise immediately on incubation and reached a peak at about 10 min., whereas no significant level of activity could be detected in TTP until 10 min. after the start of the incubation. Thereafter, the specific activity of the TTP increased steadily up to 1 hr. while that of the TDP declined.

These results strongly suggest that the formation of TTP from TdR proceeds through TMP and TDP.

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Acetolactate Metabolism and the Presence of a Dihydroxy Acid Dehydratase in Micro-organisms

By R. L. WIXOM (introduced by D. D. WOODS). (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

As the biosynthetic pathways of lysine and ornithine are different in bacteria and moulds, a survey was undertaken to ascertain the similarity or dissimilarity of valine and isoleucine biosynthesis in various biological systems (Wixom, Wikman & Howell, 1961). Nutritional requirements and the presence of dihydroxy acid dehydratase, EC 4.2.1.9, were again used to detect the capacity for valine and isoleucine formation. Log-phase cells of *Bacillus subtilis*, *Acetobacter rancens*, *Staphylococcus aureus* and *Proteus morganii* were ruptured by sonic oscillation or lysozyme action, centrifuged and assayed for the dehydratase. Each extract catalysed keto acid formation from DL-$\alpha\beta$-dihydroxyisovaleric acid, and at a slower rate from DL-$\alpha\beta$-dihydroxy-β-methylvalerate, known intermediates respectively in valine and isoleucine biosynthesis in *Neurospora crassa* and *Escherichia coli*. The dehydratase specific activity was greater when each of the four organisms was grown on valine- and isoleucine-deficient media than on crude broth or complete amino acid mixtures.

The first three organisms above, along with dehydratase-containing *Aerobacter aerogenes* and *Serratia marcesens* (Wixom *et al.* 1961), are Voges–Proskauer positive organisms. Previous investigations have demonstrated α-acetolactate decarboxylase in extracts of *A. aerogenes*, *S. marcesens*, *B. subtilis* and *S. aureus* (Juni, 1952), and α-acetolactate formation in *P. morganii* (Kobayashi & Kalnitsky, 1954) and *A. rancens* (De Ley, 1959). Thus these results re-emphasize the role of acetolactate in valine synthesis, as well as acetoin formation as an end-product of carbohydrate fermentation (cf. Halpern & Umbarger, 1959).

The dehydration of the two dihydroxy acids was also catalysed by cell-free extracts of Actinomycetales (*Nocardia corallina* and *Streptomyces aureofaciens*), Basidomycetes (*Psalliota campestris*) and Fungi Imperfecti (*Aspergillus niger*, *Penicillium chrysogenum* and *Cephalosporium* C.M.I. 49, 137). The dehydratase findings are consistent with the pattern of [1-^{14}C]acetate incorporation into valine in *P. chrysogenum* (Stevens & De Long, 1958). Thus, with one possible exception, the results to date provide necessary, but not sufficient, evidence for the hypothesis of a similar valine and isoleucine biosynthetic pathway in higher plants and the tested micro-organisms that can grow on ammonia nitrogen as the main nitrogen source.

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The Physical Properties of Citrate Oxaloacetate-Lyase from *Aerobacter aerogenes*

By T. J. BOWEN and L. J. ROGERS. (*Department of Biochemistry, University of Leeds*)

Citrate oxaloacetate-lyase (citrate lyase, citrase) catalyses the breakdown of citrate to oxaloacetate and acetate; it requires bivalent metal ions such as Mg^{2+}. It may be induced in *Aerobacter aerogenes* (Dagley & Dawes, 1953, 1955), in *Streptococcus faecalis* (Gillespie & Gunsalus, 1953; Smith, Stamer & Gunsalus, 1956) or in *Escherichia coli* (Grunberg-Manago & Gunsalus, 1953; Dagley, 1954).

The enzyme from *E. coli* has been purified (Bowen & Siva Raman, 1960) and also that from *A. aerogenes* (Siva Raman, 1961). For the present study the method of Siva Raman (1961) was slightly modified and involved the use of streptomycin sulphate, C_γ alumina gel, ammonium sulphate fractionation and chromatography on diethylaminoethylcellulose.

The purity of the enzyme was established by analytical ultracentrifugation and electrophoresis at pH 7·1 and 6·0. Diffusion coefficients were measured by the area–height method in a synthetic boundary ultracentrifuge cell. A partial specific volume of 0·73 was assumed (Kragh, 1961). Values of $D_{20,w} = 4{\cdot}16 \times 10^{-7}$ cm.^{2}sec.$^{-1}$ and $S^0_{20,w} = 16{\cdot}2$ Svedberg units were obtained. These data are consistent with those for a nearly spherical hydrated molecule. The molecular weight was found to be $3{\cdot}40 \times 10^5$ by the Archibald method and $3{\cdot}18 \times 10^5$ by sedimentation–diffusion and was consistent with the geometric series for enzymes described by Wright (1962).

A study of the factors governing the stability of the enzyme has been carried out. Extensive degradation to low-molecular-weight material is caused by 5 M-urea.

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Immunological Examination of the Soluble Antigens of Human Brain and their Modification by Proteolytic Enzymes

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This work is an attempt to identify tissue-specific antigens in human brain, soluble in aqueous solutions and demonstrable by precipitin techniques. One such antigen, a lipid, was described by Ross (1960) as released from rat-brain suspension by the action of trypsin.

Rabbits were immunized by the intramuscular injection of whole brain suspension (20% w/v) and Freund adjuvant. Response to this antigen was poor and even after several courses of injections the precipitin titre against a standard saline extract of brain was only 1/32. The same serum had a titre of 1/128 using an extract of human kidney of equivalent concentration. Diffusion in agar (Ouchterlony, 1949) with extracts of brain, heart, liver and kidney showed several lines for each tissue, with reactions of identity between the extracts. Absorption with red blood cells or heating to 56° for 30 min. produced no material change. The antiserum also reacted with human serum, probably as a consequence of contamination of the original brain suspension with plasma proteins. Further tests using the whole tissue suspension, either homogenized or ultrasonically disintegrated, give the same reactions as the extracts. The reactions of successive saline extracts showed that all the diffusible precipitating antigen can be extracted with saline. Absorption of the antiserum with liver or kidney extracts reduced the precipitin titre to zero and abolished most of the lines in agar-gel diffusion. There appeared to be one very faint and diffuse line reacting with brain only, in a position that would normally be masked by the multi-specific cross-reactions.

Extracts of brain digested with trypsin for periods up to 3 hr. at 37° and pH 8 gave results ranging from diffuse precipitation to nil on agar-gel diffusion. Precipitin titration was not possible under these conditions. A series of comparison experiments using trypsin at pH 8 and ficin and papain activated with dimercaptopropanol at pH 7·6 again showed some variation in intensity of reaction with time of digestion. Differences in the products from the three enzymes were not apparent in these tests. No new lines were produced by any of the enzyme treatments. Brain extracted with saline before enzyme treatment gave markedly weaker reactions. This suggests firstly that precipitating antibodies are formed only against the water-soluble components of human brain and secondly that human and rat brain react in a different manner, both with enzymes and as antigens.

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The Electrophoresis of Histones in Polyacrylamide Gels

By H. J. Cruft. (*Department of Biochemistry, University of Edinburgh*)

The interpretation of zone-electrophoretic patterns of histones in starch gels is complicated by interactions between components themselves and with the starch. Thus the order and separation of components on starch-gel electrophoresis (Neelin & Neelin, 1960; Johns, Phillips, Simson & Butler, 1961) bear little relation to results obtained with moving-boundary electrophoresis (Cruft, Mauritzen & Stedman, 1957).

Electrophoresis was attempted in a medium completely free from dissociable carboxyl groups, assuming that combination with such groups vitiates experiments in starch gels at any but very low pH's. Since electrophoresis in polyacrylamide gels has been reported (Raymond & Wang, 1960) this medium was used.

Best results are obtained with 10% acrylamide gels. Acrylamide (2 g.) and 40 mg. *NN'*-methylenebisacrylamide are dissolved in 20 ml. buffer

(usually 0·01 M-acetate). Immediately before pouring 0·2 ml. of 10% (v/v) solution of *NNN'N'*-tetramethylethylenediamine in ethanol followed by 0·2 ml. of freshly prepared 10% (w/v) aqueous ammonium persulphate are added with gentle stirring. The solution is poured into a Perspex trough (15 cm. × 5 cm. × 0·3 cm.). The lid is put on expelling excess of liquid and any air bubbles. The gel sets rapidly, is completely transparent and ready for use in one hour. Samples are applied on filter-paper strips inserted in cut slits and thin plastic sheet is used to cover the gel during the run which lasts three hours, at 3 v/cm.; no cooling is applied. The whole gel is stained with naphthalene black 10B in water–methanol–acetic acid (5:5:1) and cleared with the same solvent. Finally the bands are photographed using Ektachrome to give transparencies for detailed study.

Unfractionated ox-thymus histone gives a pattern comparable with that obtained on moving-boundary electrophoresis but with much higher resolution and is very different from results with starch-gels. In particular, the α or 'high-lysine' histones move fastest and resolve into four faint turquoise bands ahead of the two major blue bands.

Aggregates of β or 'high-arginine' histone if present do not appreciably enter the 10% gels (they will enter 5% gels but the resolution of other components is much poorer).

A study of fractionated histones at present in progress shows the presence of additional components and permits the identification of interaction products.

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Complex Formation between Human Cartilage Chondromucoprotein and Human Plasma Components

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Addition of chondromucoprotein to human plasma, after removal of euglobulins, has been found to result in the precipitation of a complex containing lipoprotein and fibrinogen. Kerby, Taylor & Langley (1961) observed a similar effect following the addition of pure chondroitin sulphate. The complexes formed by the addition of chondroitin sulphate and chondromucoprotein (complexes A and B, respectively) have been analysed and some properties are reported. The chondromucoprotein used was a highly viscous fraction (subfraction 1) separated by chromatography on diethylaminoethylcellulose from a crude extract prepared from human cartilage at pH 10·3 (Anderson, 1962*a*).

Chondroitin sulphate or chondromucoprotein (1 mg.) added to human plasma (1 ml., after dilution with 9 ml. water, adjustment to pH 4·4 and centrifugation) resulted in the precipitation of 9·8 and 7·7 mg. of complexes A and B, respectively, containing 50% of the plasma cholesterol originally present. Maximum precipitation of A and B occurred at pH 5·0 and 5·6, respectively, appreciable precipitation of B occurring at pH 6·5. A and B, isolated at pH 4·4, contained: acetone-extractable lipid, 21, 28; cholesterol, 10·7, 13·1; total N, 12·0, 11·3; protein, 73, 69; mucopolysaccharide, 6, 3; hexosamine, 3·3, 3·1; hexose, 2·3, 3·1; sialic acid, 0·7, 0·9%, respectively. Less than 0·1% of hydroxyproline was present.

The caseinolytic and fibrinolytic methods of Norman (1957) and Anderson (1962*b*, *c*) showed the presence of plasminogen and a plasmin substrate and incubation of complex B with streptokinase caused proteolysis. Antisera prepared in rabbits against complexes A and B reacted with B and A, respectively, and also with human fibrinogen. Three other antigenic components common to both A and B were also detected. Paper electrophoresis (pH 8·6) showed only globulins (α, β and γ) and β-lipoproteins present.

These complexes may be related to the 'fibrinoid' deposits found in 'collagen' and vascular diseases and also in rabbits following plasminogen injection (Lack, Anderson & Ali, 1961; Lack, 1962). Depolymerized chondromucoprotein, released from cartilage by plasmin, may be transported to tissues in association with plasma lipid and fibrinogen, where precipitation as 'fibrinoid' may occur. Data presented here indicate that small amounts of chondromucoprotein will complex with, and render insoluble, relatively large amounts of lipid and fibrinogen. 'Fibrinoid' deposits are at present being investigated for components similar to complex B.

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Plasma Lipids and Proteins in Rats Given Thrombogenic and Atherogenic Diets

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Thrombosis in the cardiac chambers and aorta can be produced in the rat by feeding a diet containing 40% butter (group 1), 5% cholesterol, 2% cholic acid and 0·3% thiouracil (Thomas & Hartroft, 1959). If arachis oil (group 2) is substituted for butter, no thrombosis is seen, but atherosclerosis of the proximal aorta and coronary arteries occurs (Gresham & Howard, 1960). Groups of rats have been given the above diets for four weeks, and then killed. Results of lipid and protein analyses have been compared with animals receiving a control diet (group 3).

Similar changes were observed in both experimental groups, but the magnitude of the change was generally much greater in the group receiving butter. The very large increase in plasma cholesterol previously reported (Howard & Gresham, 1960) was also accompanied by elevated phospholipids (group 1, 1220; group 2, 550; group 3, 110 mg./100 ml.). The percentage composition of phospholipids was also markedly changed. Lecithin was increased and lysolecithin diminished (% composition: group 1—lecithin 69, sphingomyelin 15, lysolecithin 15; group 2—lecithin 54, sphingomyelin 22, lysolecithin 25; group 3—lecithin 45, sphingomyelin 16, lysolecithin 35). These changes are similar to those seen in essential fatty acid deficiency (Howard *et al.* 1962).

Analyses of total fatty acids by gas–liquid chromatography showed that the greatest change occurred in the percentage arachidonic acid which was reduced to one-sixth of normal. Since the total fatty acids were greatly elevated (group 1—20-fold, group 2—10-fold), the net amount of this acid was not, however, decreased. A lowered percentage of arachidonic acid was unexpected, since the highest proportion of fatty acids were derived from the cholesterol esters, and in the rat, cholesterol is normally esterified with 50% arachidonic acid (Swell, Field & Treadwell, 1960). Oleic acid was also increased. (Percentage fatty acids: group 1—oleic 47, arachidonic 1·4; group 2—oleic 62, arachidonic 1·7, group 3—oleic 29, arachidonic 8·1).

The total plasma protein concentration was also slightly elevated (% protein: group 1, 5·9; group 2, 6·6; group 3, 5·4), and accompanied the increased level of fibrinogen, previously reported (Davidson, Howard & Gresham, 1962). Results of paper-electrophoresis studies were discussed.

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The Effects of Age and of Early Atheromata on the Intimal Lipids in Men

By Elspeth B. Smith. (*The Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

One of the many unknown factors in atherosclerosis is the source of the lipid which accumulates in the arterial wall. This investigation is an attempt to assess separately the effects on intimal lipids of age and of the earliest stages of atherosclerosis.

In macroscopically normal intima the concentration of esterified cholesterol is highly correlated with age. At 10 years there is about 3 mg./g. dry tissue, which increases to 35 mg./g. at age 65 (correlation coefficient $r = 0{\cdot}935$; $P < 0{\cdot}01$). Free cholesterol increases more slowly from 10 mg./g. at age 10 by 1 mg. per decade ($r = 0{\cdot}788$; $P = 0{\cdot}01$). Phospholipid and triglyceride are more erratic, but tend to fall with age and consequently the composition of the total initimal lipid changes; in three children (average age 11) cholesterol ester (C.E.) comprised 8·9% of the lipid, but rose to 48·5% in men over 50. For comparison, the S_f 0–12 lipoprotein of normal serum contains 58% of C.E. and shows no consistent change in composition with age (Smith, 1962).

The two major fatty acids of the cholesterol esters are oleic (18:1) and linoleic (18:2). The proportion of oleic acid remains rather constant at about 29% of the total fatty acid over a wide age range, but linoleic acid increases from 17% at age 10 to 40% at age 65 ($r = 0{\cdot}911$; $P < 0{\cdot}01$). In the S_f 0–12 lipoprotein there is about 47% linoleic acid and no clear trend with age.

The 'fatty streak' is the earliest clearly defined atheromatous lesion; in it the ester cholesterol may increase tenfold, and rise to 60% of the total lipid. A striking change occurs in the composition of the C.E. fatty acids; oleic acid rises to about 48% while linoleic acid falls to 16% and the ratio of eicosatrienoic to arachidonic acids rises from 0·25 in

normal tissue to 1·0 in the fatty streaks. It is difficult to visualize how this cholesterol ester could be derived from plasma; the fatty acid pattern is suggestive of essential-fatty acid (E.F.A.) deficiency (Holman, 1960), but clearly no generalized deficiency can exist where the cholesterol ester of adjacent normal intima may contain 40% linoleic acid. In experimental animals E.F.A. deficiency is exacerbated by feeding cholesterol, and it is possible that these intimal cells are esterifying cholesterol so rapidly that they have exhausted their supply of E.F.A.

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The Fine-Structure of Amylose

By O. KJÖLBERG and D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

Peat, Pirt & Whelan (1952) showed that the action of β-amylase on amylose was incomplete, the conversion into maltose being about 70%. This observation has since been confirmed in several laboratories but the nature of the barrier to β-amylase was not established (cf. Manners, 1959). We have now re-examined the possibility that the anomalous linkages in amylose were a small number (perhaps 0·1%) of α-1,6-glucosidic inter-chain linkages similar to those present in amylopectin.

The outermost α-1,6-inter-chain linkages in amylopectin are hydrolysed by yeast isoamylase causing a marked increase in β-amylolysis limit and a decrease in viscosity (Gunja, Manners & Khin Maung, 1961). The effect of isoamylase on these properties of four samples of potato amylose and on oat amylose has been examined. The enzyme was isolated from baker's yeast and shown to be free from α-amylase, whilst the substrates, prepared with the assistance of Mr J. R. Stark, had β-amylolysis limits ranging from 76 to 97% and were free from amylopectin.

In all experiments there was an increase in β-amylolysis limit and a limited fall in specific viscosity, the extent of which paralleled the differences in β-amylolysis limit. For example, with one potato amylose the β-amylolysis limit increased from 97 to 100% whilst the specific viscosity fell by only 3% whereas with oat amylose a 22% increase in β-amylolysis limit (from 76 to 98%) was accompanied by a 19% decrease in specific viscosity.

These results suggest that some amylose molecules are slightly branched, in agreement with certain physicochemical observations (Potter & Hassid, 1951; Kerr & Cleveland, 1952). On this basis, incubation of amylose β-limit dextrin with isoamylase should cause an increase in β-amylolysis limit without a change in specific viscosity, and this has been observed experimentally.

Since isoamylase appears to be specific for the hydrolysis of α-1,6-glucosidic linkages, the present findings characterize the barriers to β-amylase in amylose. This implies that in the biosynthesis of starch the action of Q enzyme is sufficient to account for the synthesis of all glucosidic linkages, other than the repeating α-1,4-type. It is not therefore necessary to postulate either the existence of additional starch-metabolizing enzymes or that those already known can by a chance error synthesize an anomalous linkage.

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Structural Lipoproteins of the Mucosal Cell

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Sterols undergoing absorption are rapidly mixed with endogenous sterols throughout the mucosal cell. 60–70% of the absorbed sterol in the cell is associated with the mitochondria and microsomes and previous studies suggested that this fraction is taken up by the structural lipoproteins of these cell components (Glover & Green, 1956).

Mitochondria and microsomes from the intestinal mucosa of fasted guinea pigs were extracted twice with 0·15M-NaCl (Dallam, 1958). They were then either suspended in 0·15M-NaCl and treated with ultrasonic vibrations (20 kcyc./sec.) for 20 min. or homogenized with one of the following: (1) 0·5% sodium deoxycholate; (2) 1% digitonin; (3) a solution of mixed detergents (Green, Tisdale, Criddle, Chen & Bock, 1961). After centrifuging at 105 000*g* for 20 min. the total nitrogen, total sterol, 7-dehydrocholesterol and phospholipid contents of the soluble and insoluble fractions were determined.

Sodium deoxycholate treatment rendered soluble a greater proportion of all components studied than any other method. From the saline-extracted mitochondria and microsomes, 80–85% of the total nitrogen, 75% of the total sterol and 85–95% of the phospholipid were extracted.

Paper electrophoresis at pH 9·2 (0·1 M-borate buffer) of the deoxycholate extracts revealed one protein band migrating towards the anode. Cholesterol and phospholipid were associated with the protein zone but 7-dehydrocholesterol remained at the origin. This may reflect a difference in the ability of the two sterols to enter deoxycholate micelles or it could mean that the two sterols occur in different lipoproteins.

During absorption of 7-dehydrocholesterol, the ratio of total sterol to 7-dehydrocholesterol in deoxycholate extracts of mitochondria and microsomes falls from 15–16:1 to 6–9:1. The amount of sterol rises in the mitochondrial extracts from 0·3 to 0·55 mg./mg. nitrogen, and in the microsomal extracts from 0·5 to 0·85 mg./mg. nitrogen.

Despite the higher sterol content of the organelles during absorption, the same percentages of the total were removed by saline and deoxycholate solutions as from controls.

These results confirm previous findings that the absorbed sterols are bound in the same way as the endogenous ones in the structural lipoproteins of the mitochondria and microsomes.

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The Essential Role of Noradrenaline in Corticotrophin-Induced Fatty Acid Mobilization

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Corticotrophin (ACTH) is well known to stimulate the release of free fatty acids (FFA) from the rat epididymal fat pad incubated *in vitro* (Shotz, Masson & Page, 1959; Vaughan, Steinberg & Shafrir, 1959). Adrenaline and noradrenaline also increase lipid mobilization *in vitro* (White & Engel, 1958) and *in vivo* (Shafrir, Sussman & Steinberg, 1960). The *in vitro* fat-mobilizing effects of ACTH and adrenaline are both antagonized if the fat pad is pretreated with an adrenergic blocking agent (Shotz & Page, 1960). This suggests that catecholamines may play an intermediary role in ACTH-induced release of FFA, especially since recently noradrenaline has been shown to be present in the adipose tissue of several species (Smith, Paoletti & Brodie, 1962). The data obtained using adrenergic blocking agents is not entirely satisfactory since these agents are somewhat non-specific in their action.

To study this possible role for noradrenaline in fat, we have investigated the lipid responses to ACTH after depleting the catecholamine from its normal storage sites in adipose tissue, using the alkaloid reserpine. Reserpine depletes noradrenaline from adipose tissue (Paoletti, Smith, Maickel & Brodie, 1961) as well as from many other tissues (Brodie, Olin, Kuntzman & Shore, 1957; Carlsson & Hillarp, 1956).

Epididymal fat bodies were removed from normal and reserpine-treated rats and incubated *in vitro* with ACTH and the FFA released determined (Dole, 1956). The hormone elicited a marked increase in the total amount of FFA released when added to the fat pads taken from normal animals. However, there was no significant effect on the pads taken from reserpine-treated rats. The addition of small quantities of noradrenaline (0·02–0·05 μg.) to the incubation medium before adding the ACTH largely restored the effect of the polypeptide. These low doses of noradrenaline alone showed little effect in stimulating FFA release.

Depot ACTH *in vivo* also induces mobilization of FFA and deposition of triglycerides in the liver. These responses to the hormone were abolished if the animals were depleted of catecholamines by pretreatment with reserpine. When the reserpinized animals were infused intravenously with noradrenaline the effects of ACTH on plasma FFA and liver triglycerides were found to be largely restored. These data are consistent with the view that ACTH releases fatty acids from adipose tissue by a mechanism involving noradrenaline.

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The Degradation of Apoferritin into Subunits

By T. HOFMANN and PAULINE M. HARRISON. (*Department of Biochemistry, University, Sheffield* 10)

It has been suggested that apoferritin (mol.wt. 480 000) consists of a number of chemically identical subunits (Harrison, 1959; Harrison & Hofmann, 1962). Unlike some viruses which readily degraded into subunits in the presence of urea or sodium dodecyl sulphate (SDS) (Bawden & Pirie, 1940; Hersh & Schachman, 1958), however, native apoferritin was completely resistant to these agents. It has now been found that when neutral salt-free solutions of apoferritin are frozen or freeze-dried and the product is dissolved in SDS solutions it readily degrades into subunits with a sedimentation coefficient $S^0_{20,w} = 2{\cdot}5$s ($S^0_{20,w}$ for apoferritin = 17·6s; Rothen, 1944). Only one intermediate break-down product ($S_{20,w} = 5{\cdot}5$s) has been found. The degradation is a function of the ratio SDS:protein, but appears to be independent of concentration and temperature. At SDS:protein ratios of 1:3 and higher an essentially homogeneous SDS–protein complex is formed, which at a ratio of 1:2 has a sedimentation coefficient $S^0_{20,w} = 2{\cdot}5$s.

Incubation of native apoferritin at pH 12·5 leads to a slow degradation. Ultracentrifuge patterns show only two distinct peaks, with $S_{20,w} = 16$s and 2·1s, both of which appear homogeneous during the early stages of degradation. This indicates that the degradation is due to splitting into subunits and not because of proteolysis.

Preliminary molecular-weight determinations were made on the SDS–apoferritin complex, both from measurement of sedimentation and diffusion coefficients and by the approach to equilibrium method (Archibald's principle; Schachman, 1957). The composition of the complex was determined by studying the SDS binding by equilibrium dialysis. A complex consisting of 67% apoferritin (freeze-dried) and 33% SDS was mainly used. The apparent molecular weight as determined by the Archibald method showed a strong dependence on concentration, decreasing by a factor of about 3 between infinite dilution (41 000) and a concentration of 1% (12 000). This is due to a very strong concentration dependence of the sedimentation coefficient (which decreases with increasing concentration) and of the diffusion coefficient (which increases with increasing concentration). The molecular weight calculated from $S^0_{20,w}$ and $D^0_{20,w}$ is about 38 000, in good agreement with the Archibald value at infinite dilution. The molecular weight of the protein part of the complex is about 25 000–27 000 suggesting that the molecule is made up of 18–19 subunits. This agrees with the evidence from end-group analysis (20 subunits; Mainwaring, 1962) and tryptophan content (21 subunits; Harrison, Hofmann & Mainwaring, 1962), with the results of fingerprinting (Harrison & Hofmann, 1962), and with recent X-ray diffraction studies (Harrison, 1962) which suggest that the molecules may have an arrangement of 20 subunits at the apices of a pentagonal dodecahedron.

One of us (P.M.H.) would like to thank the Medical Research Council for a personal research grant.

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Fractionation of Soluble Tissue Proteins from Brain

By D. L. MOULD and A. McL. DAWSON. (*Animal Diseases Research Association, Moredun Institute, Edinburgh*, 9)

In a current study of neuropathological conditions in sheep data on various constituents of sheep brain tissue have been obtained and the serological response of rabbits to inoculation of brain-tissue preparations investigated. The immunological response to nervous tissue is poor and methods of fractionating and concentrating soluble proteins from brain were considered to be of use in both attempting to characterize the antigenic components and enhance the possible antibody reaction. The general procedure adopted has been the extraction of frozen ground tissue in 20% NaCl (Lebaron & Folch, 1959). The extracted proteins have been fractionated by salt precipitation or zone electrophoresis followed by chromatography

on diethylaminoethylcellulose columns (Burston, Tombs & Maclagan, 1961) and the constituents of the various fractions examined by starch-gel electrophoresis.

Many of the components are unstable and tend to form insoluble precipitates during concentration. Perevaporation in dialysis sacs has been the most satisfactory procedure (cf. Tombs, Cooke, Burston & Maclagan, 1961). About 30–40% is irreversibly adsorbed on the cellulose columns. The various fractions obtained are, in general, still very complex mixtures.

Antigenic components in the fractions have been examined by microimmunoelectrophoresis and have been located within the starch-gel patterns by laying a starch-gel strip or a thin layer of agar parallel to a trough cut in the agar containing antiserum (Press & Porter, 1962). Partial purification and concentration of the antigens has been achieved and further problems of isolation and characterization were discussed.

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Analysis of Sterols in Blood Serum by Gas–Liquid Chromatography

By C. J. W. Brooks and J. S. Young. (*Medical Research Council, Clinical Chemotherapy Research Unit, Western Infirmary, Glasgow*)

The work of E. C. Horning and co-workers (*inter al.* VandenHeuvel, Sweeley & Horning, 1960*a*, *b*) indicated the value of low concentrations of stationary phase in gas–liquid chromatography of steroids. These authors' methods have now been applied to the determination of cholesterol in serum, using the Pye Argon Chromatograph modified for syringe injection. The column (4 ft. × 3·5 mm. int. diam.), packed with 'siliconized' Gas-Chrom P (100–140 mesh) coated with 1% SE-30, is operated at 225° (argon flow rate 30 ml./min.).

Saponification of serum (0·20 ml.) and extraction of cholesterol are achieved by a modification of the procedure of Abell, Levy, Brodie & Kendall (1952). Aliquots of the extract are taken for (i) thin-layer chromatography to assess the completeness of hydrolysis, (ii) gas–liquid chromatography and/or (iii) spectrophotometric estimation. For (ii), an aliquot containing 0·1–0·4 mg. of cholesterol is mixed with 1·00 ml. of cholestane solution (100 μg./ml. in chloroform) and the solvents are blown off by nitrogen. The residue is dissolved in chloroform and an aliquot (1–4 μg. of cholesterol) is injected. The retention time of cholesterol is 23–24 min.

The ratio of concentrations (cholesterol:cholestane) is evaluated from the ratio of the respective peak areas or heights: both parameters afford approximately linear relationships over the range of concentrations described. Calibration is effected by processing standard solutions.

The validity of the method is supported by the reproducibility of repeated estimations (performed concurrently or at intervals) and by the satisfactory increments found when cholesterol is added to the serum–alkali mixture. The cholestan-3-ols would be estimated as cholesterol, but in other respects the method is specific. We have confirmed that desmosterol present in the serum of patients treated with 'MER-29' may be detected, and approximately determined, by its peak at a retention time 10% beyond that of cholesterol (Steinberg, Avigan & Feigelson, 1961).

The full sensitivity of gas–liquid chromatography is not realized in this procedure, in which less than 1% of the sample is finally measured. The aliquot for injection could be increased, but a method for introducing entire samples without solvent would be advantageous for the analysis of 2–4 μg. of sterol (i.e. 1–2 μl. of serum), and would also permit faster analysis by reduction of retention times.

We are indebted to Professor E. C. Horning (Houston, Texas) for his advice and encouragement.

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A Possible Relationship between the Specificity of the Intestinal Absorption of Sterols and their Capacity to form Micelles with Phospholipid

By P. G. Barton and J. Glover. (*Department of Biochemistry, The University of Liverpool*)

Cholesterol is dispersed in bile as a micellar complex with phospholipids and the conjugated dihydroxycholanic acids in the molecular proportion 1:4:7 (for the ox) along with a trace of muco-

protein (approx. 1%) (Desai & Glover, 1961). Again, cholesterol is always associated with phospholipid in tissue lipoproteins. Furthermore, bile salts can interact with serum lipoproteins to form a negatively charged micellar complex containing free cholesterol and phospholipid (Glover & Joo, 1961).

The capacities of various sterols with small differences in molecular structure to pack with phospholipids in forming molecular aggregates such as lipoproteins or micelles is probably important for their transport and absorption across the intestine.

It has been shown that during the early stages of absorption of fat, the level of bile salt in the human intestine (Sjövall, 1959) is above the critical micellar concentration (CMC) for the formation of micelles of fatty acid with lysolecithin or monoolein and bile salts (Hofmann, 1961). Micellar complexes may therefore be involved in fat absorption.

Cholesterol and ergosterol have now been examined for their capacity to form micelles comparable to those found in bile. Bashour & Bauman (1937) succeeded in dispersing a little cholesterol with solutions of bile salts >1% or 20 mM. We have been unable to disperse appreciable amounts of cholesterol with lower concentrations of taurodeoxycholate (3–10 mM) such as would be encountered in the intestine during digestion of lipid. On the other hand, lecithin readily disperses cholesterol at a CMC of 0·4 mM in phosphate buffer (0·1 M, pH 6·8) and the molecular proportions of cholesterol to phospholipid in the micelle is 1:10. The addition of taurodeoxycholate to this system can then markedly improve the proportion of cholesterol to lecithin in the new micelles, which reaches its maximum when the molar concentration of bile salt is double that of lecithin (the respective proportions in the new micelle are 1:3·5:7). A further increase in bile salts up to 6 mM does not bring about the dispersion of any more cholesterol. This can only be accomplished by supplements of lecithin. In dilute solution therefore lecithin and not bile salt is the more effective agent for dispersing cholesterol.

Ergosterol, on the other hand, does not form a micelle so readily with lecithin and requires a CMC of 1·5 mM to be reached. Furthermore, in this micelle the proportion of ergosterol to lecithin is only 1:18. This inferior capacity to pack with lecithin in molecular aggregates may partly explain its poor absorption relative to cholesterol.

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The Interaction of Polycyclic Hydrocarbons and Nucleic Acids

By E. Boyland and B. Green. (*Chester Beatty Research Institute, Fulham Road, London, S.W. 3*)

The interaction of polycyclic hydrocarbons and purines (Boyland & Green, 1962) results in increased solubility of the hydrocarbons in aqueous purine solutions. Interactions of these hydrocarbons with nucleic acids, following the report that benzo[a]pyrene was slightly more soluble in DNA solution than in water (Booth, Boyland, Manson & Wiltshire, 1951), have been studied by techniques previously described (Boyland & Green, 1962). The DNA (calf thymus) and RNA (rat liver) were provided by Professor J. A. V. Butler.

Both DNA and RNA exerted a definite solubilizing action on the carcinogenic benzo[a]pyrene and on the non-carcinogenic pyrene. A 0·05% solution of DNA in water solubilized 3–7 μM-benzo[a]pyrene and about 22 μM-pyrene. In the presence of NaCl (0·01 M or 0·1 M) the amount of benzo[a]pyrene solubilized was reduced considerably (<1 μM), whereas the solubilization of pyrene was less affected (6 μM). The rigid helical structure of DNA appears to be important for this binding since heat-denatured DNA (and RNA) had less than one-quarter of the solubilizing activity of native DNA.

Pyrene 'bound' to DNA in aqueous solution displayed a bathochromic shift in the u.v. absorption spectrum (maxima 329 and 345 mμ as compared with 319 and 335 mμ in water) and depression of the maxima. These spectral changes were not observed in ethylene glycol or formamide.

DNA quenched the fluorescence of both benzo[a]pyrene and pyrene in aqueous solutions but not (at comparable concentrations) in formamide or methanol. RNA also quenched the hydrocarbon fluorescence in aqueous solutions but was less efficient than DNA. The binding forces involved in these interactions are weak, since the hydrocarbon could be removed by extracting the aqueous DNA solution with cyclohexane; they probably involve some form of 'hydrophobic bonding' (Kauzmann, 1959) to the stacked bases in the core of the DNA helix.

Work with molecular models (Boyland & Green, 1960) has shown that the hydrocarbons could intercalate between the planar base-pairs of the DNA molecule without disrupting the sugar phosphate

side-chains: the evidence at present does not distinguish between this mechanism and simple binding to the external surface of the stacked bases.

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Apparent Heterogeneity of Prostatic Acid Phosphatase

By B. K. Sur*, D. W. Moss and E. J. King. (*Department of Chemical Pathology, Postgraduate Medical School, London, W.* 12)

Acid phosphatase in concentrated aqueous extracts of human prostate gland can be separated into at least 13 active zones on starch-gel electrophoresis in citrate buffer at pH 6·2 (Sur, Moss & King, 1962). These fractions do not appear to be in dynamic equilibrium with each other, since on recovery from the gel and re-electrophoresis they maintain their relative mobilities. The acid-phosphatase activity of human semen moved under similar conditions as a broad band slightly ahead of but overlapping the fastest region of prostatic phosphatase.

The phosphatase zones of the prostatic extract were recovered from the gels in four groups according to their mobilities, and some of their properties were studied and compared with those of seminal phosphatase recovered similarly from starch-gels. In these experiments, 0·005% Triton-X-100 was added to prevent surface denaturation in the highly dilute enzyme solutions and 0·005M-EDTA to prevent inactivation by metals.

Semen acid phosphatase and all four prostatic fractions had broad pH optimum curves centred around pH 5·5 (substrate, disodium β-naphthyl phosphate) and at this pH the apparent Michaelis constants towards this substrate of all the fractions were not significantly different. Their thermal stabilities at 47° and pH 6·2 were also indistinguishable.

These results suggested the possibility that the multiplicity of electrophoretic fractions of prostatic acid phosphatase might be due to polymerization or complex formation of a single enzyme identical with that of semen acid phosphatase. Attempts were made therefore to modify the electrophoretic pattern by the use of agents which might be expected to cause depolymerization or reversal of complex formation. The prostatic extract was treated with (i) butanol which dissociates lipoprotein–phosphatase complexes (Morton, 1950), (ii) a powerful protease (*Streptomyces griseus* protease), in case complexing with other proteins was involved, and (iii) EDTA, to dissociate possible metal-bound complexes. None of these agents altered the electrophoretic pattern. Although it appears likely that the multiple electrophoretic zones are different forms of a single enzyme, the way in which they arise remains obscure.

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Phosphorus Compounds Released by Skin *in vitro*

By H. J. Yardley and G. Godfrey. (*Medical Research Council Unit for Research on the Experimental Pathology of the Skin, University of Birmingham*)

Phosphorus compounds of skin have been studied by Brooks, Lawrence & Ricketts (1959). Using similar methods we have examined the medium upon which skin has been cultured for phosphorus compounds.

Slices of guinea-pig ear skin weighing about 5 mg. were incubated at 37° in 0·5 ml. medium (Cruickshank & Trotter, 1956) consisting of horse serum (5 parts), Krebs–Ringer phosphate (Krebs, 1933) (3 parts), 0·2M-glucose (1 part), streptomycin 500 μg./ml. (1 part) and [^{32}P]orthophosphate 200 μc/ml. After 24 hr., the skin was carefully transferred to 0·1 ml. 'cold' medium consisting of Krebs–Ringer phosphate (8 parts), 0·2M-glucose (1 part) and streptomycin 500 μg./ml. (1 part). After a further 4 hr. incubation at 37°, 0·025 ml. medium was subjected to two-dimensional paper chromatography in propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (60:30:10, by vol.) followed by picric acid–*tert.*-butanol–water (4:80:20, w/v/v). Labelled phosphorus compounds were detected by radioautography. After each incubation, the medium was tested for sterility and the presence of glucose.

Apart from an intense spot due to orthophosphate, three strong spots were seen on the radioautograph together with some weaker ones. The strong fast-moving spot in the alkaline solvent was identified as glycerylphosphorylcholine by co-chromatography with the product of mild alkaline hydrolysis of egg lecithin, and also by co-chromatography of its hydrolysis product with α-glycerophosphate. Near this strong spot were three weaker ones, two of which have been provisionally identified as glycerylphosphorylethanolamine and

glycerylphosphorylserine on the basis of chromatographic and ionophoretic measurements. A second strong spot was identified as α-glycerophosphate by co-chromatography with an authentic sample and by repeated co-crystallization with barium α-glycerophosphate.

The third strong spot was resolved into two compounds by further paper chromatography in phenol–aq.NH_3 (sp.gr. 0·88)–water (80:1:19, by wt.). The fast-moving component was shown to be phosphorylcholine by co-chromatography with an authentic sample and by measurement of its rate of hydrolysis in N-HCl at 100°. Using similar methods the slower-moving component was shown to be phosphorylethanolamine.

In view of the precautions taken to exclude compounds originating from damaged cells it is suggested that the compounds described have either passed through the cell membrane or, alternatively, have been formed on the cell surface. The relationship of the identified compounds to phospholipid metabolism suggests that the second hypothesis might be more correct.

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The Metabolism of Isoniazid in Rats and Mice

By J. G. Chalmers. (*Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow, C.* 3)

It is possible that the efficacy of isoniazid (INH) in the therapy of tuberculosis is related to the pattern of metabolism of the drug. The major metabolites of INH, acetyl-isoniazid (AINH) and isonicotinic acid (INA) do not possess antimycobacterial activity and humans vary in the extent to which they metabolize INH (Peters, 1960).

The metabolism of INH has been studied in animals as well as humans. Peters (1960) found that the rat and monkey converted INH largely into AINH but that the dog did not have the capacity for forming this metabolite.

Juhasz, Balo & Kendrey (1957), Mori, Yasuno & Matsumoto (1960) and Biancifiori (1961) have induced lung tumours in mice with large doses of INH (100 mg./kg. body weight compared with a therapeutic dose of 3–8 mg./kg. in humans). Lung tumours in animals other than mice have not been reported.

In the present experiments a comparison has been made of the metabolism of INH in rats and mice. It was found that the pattern of metabolism was similar in the two species with regard to unchanged INH, AINH and INA. Differences however were observed in the case of the isonicotinoylhydrazone-like substances of low R_F value. These were found to be present in rat urine but were not detected in mouse urine by paper chromatographic methods based on those of Zamboni & Defrancheschi (1954).

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Reduction of Tetrazolium Salts by Phenazine Methosulphate-Linked Oxidations

By Julia M. Sowerby and J. H. Ottaway. (*Department of Biochemistry, University of Edinburgh*)

The reduction of tetrazolium salts coupled to the enzymic oxidation of glutamate (Sowerby & Ottaway, 1961) is not complete, when the observed extinction of the reduced tetrazolium is compared with that calculated from the extinction coefficient of the chemically reduced dyes. The reduction of 2-*p*-iodophenyl-3-*p*-nitrophenyl–5-phenyltetrazolium chloride is 55% and of 3-(4,5-dimethylthiazol-1-yl)-2,5-phenyltetrazolium bromide is 75% of theoretical; in the presence of 5·7 mM-cyanide reduction is increased to about 80%. 100% reduction can be achieved either by excluding oxygen, or by increasing the concentration of glutamate dehydrogenase, which speeds up the reaction. This suggests that there is competition between oxygen and tetrazolium salts for reduced phenazine methosulphate (cf. Arrigoni & Singer, 1962). We have observed that $NADH_2$ is oxidized extremely rapidly (2–3 min.) in the presence of phenazine methosulphate and oxygen. Cyanide has no effect on this process.

A factor present in partially purified kidney glutaminase preparations (Klingman & Handler, 1958) increases the reduction of tetrazolium salts to 100% of theoretical, even in the absence of cyanide. The factor speeds up the oxidation of glutamate but it does not appear to act only on glutamate dehydrogenase since it increases the reduction of tetrazolium salts by $NADH_2$, coupled by phenazine methosulphate, from 83 to 100%.

However, the reduction of 2,6-dichlorophenol-indophenol by $NADH_2$, which is only 77% of theoretical in the absence of cyanide, is increased to 100% by cyanide alone, while the factor only increases the reduction to 88%. This suggests that the latter catalyses the reduction of tetrazolium salts by reduced phenazine methosulphate and that in the complete system both this step and the reduction of NAD by glutamate are about equally rate-limiting.

The activity is precipitated from the mitochondrial extract with glutaminase. The factor is non-dialysable, but it is not clearly an enzyme, since activity remains after boiling in dilute solutions. It is certainly not glutaminase.

Of a number of metabolic co-factors or heavy metals, with or without the addition of inert protein, only ubiquinone (6) replaces the activity of the factor.

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Inorganic Phosphate Metabolism by the Perfused Rat Heart

By A. K. Sarkar* and J. H. Ottaway. (*Department of Biochemistry, University New Buildings, Edinburgh* 8)

Insulin injection causes a fall in plasma inorganic phosphate (P_i) (Stadie, 1944), presumed to be due to an increased uptake of P_i by tissues. The isolated perfused rat heart (Bleehen & Fisher, 1954), loses P_i to a phosphate-free medium. If the perfusion is carried out in open circuit in a medium containing only 0·07 mM-P_i, it is possible to show that the net loss of P_i is reduced from 1·2 μg./g. heart/min. to 0·8 μg./g./min. by the addition of 20 mU/ml. insulin to the medium. The rate of loss is not affected by glucose, or by insulin in the absence of glucose.

This difference is not due to increased uptake of P_i. If the heart is perfused in open circuit with a low-phosphate medium containing $^{32}P_i$ of constant specific activity, the uptake can be calculated from the loss of isotope from the medium. This is justified since it can be shown that the specific radioactivity of the intracellular P_i increases linearly and reaches only 5% of that of the medium P_i after 40 min. The estimated uptake of P_i by this technique is 1·26 μg./g. heart/min. without insulin, and 1·05 μg./g./min. with insulin. The difference is not significant. A similar figure was found by Sacks & Sinex (1953) for rat diaphragm.

If the heart is perfused in open circuit with P-free medium which is collected in successive equal periods, the P_i output is found to fall off exponentially with a rate constant, k, −0·021/min. The pool size at zero time is 3·8 μmoles/g., slightly smaller than that found by Lepage (1948). If insulin is present in the perfusate, k is lower, −0·014/min., and the pool size is also lower, 2·6 μmoles/g. The intracellular P_i, estimated chemically, is not however altered by perfusion with a P-free medium containing insulin.

The loss of P_i from the heart has also been studied by following the 'wash-out' of P_i after exposure to $^{32}P_i$. In the absence of insulin a slow exponential component of the loss is found, k −0·026, with a pool size compatible with that of the intracellular P_i. If insulin is present, however, the slow component has sometimes a positive slope, and the pool size cannot be accurately calculated.

It appears that insulin may lower extracellular P_i by reducing the rate of loss from the tissues. The changes found are large enough, if applied to the whole musculature, to account for the fall of plasma P_i after insulin injection.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **418th Meeting** *of the Society was an International Meeting held in Cambridge on Thursday and Friday, 12–13 July* 1962. *The Colloquium was held on Thursday afternoon,* 12 *July, and on Thursday morning,* 12 *July, and Friday,* 13 *July, the following Communications were presented:*

COLLOQUIUM ON 'CONTROL OF PROTEIN BIOSYNTHESIS—THE ROLE OF NUCLEIC ACID'

The Nature of the Genetic Code

By F. H. C. Crick. (*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge*)

Recent work suggests that the genetic code may be a non-overlapping triplet code. The probable composition of some of these triplets has been determined by Ochoa and by Nirenberg and their co-workers. All these triplets contain at least one uracil, but for a variety of reasons it seems likely that some triplets without uracil also code for amino acids.

Whether the code is highly degenerate is at the moment a matter of opinion, but if it is, the evidence suggests that triplets coding for the same amino acid are related to each other in some way.

The Genetic Message

By S. Brenner. (*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge*)

In bacteria, genetic information for the synthesis of proteins is brought to ribosomes by a special RNA called messenger RNA. It is likely that the control of protein synthesis is exercised at the level of messenger RNA synthesis, but the molecular mechanisms of repression and induction are not understood. The experimental basis for these statements will be discussed.

A Search for a Messenger for Haemoglobin Synthesis

By Hildegarde Lamfrom. (*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge*)

In reticulocyte cell-free systems containing three cellular components—ribosomes, pH 5 fraction and supernatant—haemoglobin is synthesized which is characteristic of the cell species. In this system a supernatant fraction obtained from reticulocytes is absolutely required for the release of soluble proteins.

Cellular fractions were prepared from rabbit and sheep reticulocytes and incubated in various combinations under appropriate conditions. [^{14}C]Leucine and [^{14}C]valine were used to label the newly formed haemoglobins, which were first separated from the microsomal particles and then isolated by chromatography. In the presence of heterologous supernatant a significant amount of both types of haemoglobin was synthesized. As an extension of these experiments cellular fractions from rabbits and ducks were also tested in mixed incubation experiments. When either duck or rabbit ribosomes were incubated with supernatant from the heterologous species, an appreciable amount of protein was labelled which did not correspond in electrophoretic behaviour to the ribosomal haemoglobin. This effect was intensified by the addition of pH 5 fraction corresponding to the supernatant.

On the Frequency of Non-Genetic Amino Acid-Sequence Errors in Protein Biosynthesis

By R. B. Loftfield.* (*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge*)

In some circumstances it is clear that changes in the genetic material result in a specific substitution of one amino acid for another. In these cases, all or a large fraction of the protein molecules contain the specific error which might be described as 'genetic'. On the other hand, there should be errors in reading the genetic code, in attaching amino acids to appropriate s-RNA's, in assembling the amino acids, etc. Collectively these non-genetic errors are a kind of 'noise' in transmitting genetic information and result from the finite ability of chemical surfaces to distinguish among closely related chemical substances.

On theoretical grounds it has been estimated that valine might substitute for isoleucine 5 % of the

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time. Experimentally it has been found that an isoleucine activating enzyme shows a fifty-fold discrimination against valine in activation and a larger discrimination in the transfer of the amino acid to s-RNA. There is little experimental or theoretical basis for estimating the precision with which, let us say, three bases from DNA can prescribe a complementary trinucleotide and exclude another trinucleotide that is complementary in only two bases. However, it is self-evident that the larger the number of bases involved in the nucleotide code the greater the capacity for discrimination. If an accurate determination of the non-genetic errors in protein biosynthesis shows that errors are less frequent than would be expected from the postulated intermediate reactions, it may be necessary to modify our present ideas of protein biosynthesis.

Ovalbumin has been labelled by incubation of intact oviduct tissue with five different amino acids. After purification and chymotryptic digestion, fourteen labelled peptides were isolated. Comparison of the radioactivity due to isoleucine and valine in a single purified peptide shows that valine has substituted for isoleucine not more than once in a thousand times. Other comparisons will be described as well as reservations concerning the significance of these data.

Kinetic Studies of Ribonucleic Acid Synthesis

By B. J. McCarthy. (*Carnegie Institution, Washington, U.S.A.*)

Many recent studies of growing bacteria have been directed towards the detection of a fraction of RNA having a DNA-like base composition (D-RNA). Since there are reasons for supposing that it is rapidly synthesized, this RNA has been equated with the rapidly labelled fraction of RNA, averaging 14 s. The kinetics of incorporation of [^{14}C]uracil or ^{32}P indicate, however, that it can be considered, predominantly at least, as a precursor of the bulk ribosomal RNA. On the other hand, the base composition is not that typical of ribosomal RNA but tends toward that of the DNA. More complete investigation of this RNA fraction, some 3 % of the total RNA, shows that it is heterogeneous, consisting of about two-thirds ribosomal RNA precursor and the remainder RNA of DNA-like composition. Separation of the two components can be achieved by two different methods.

In exponentially dividing bacteria the base composition of newly formed RNA is never identical with that of the DNA, however short the exposure to ^{32}P. In fact the composition is constant up to some 3 % of a mean generation time indicating a constant proportion of ribosomal RNA and D-RNA in the labelled 14 s fraction. Changes in overall composition occur only as a result of the flow of label out of the 14 s fraction into stable ribosomal material. The rate of synthesis of the D-RNA fraction seems, therefore, to be approximately half that of the stable RNA. This rate of synthesis of a fraction of RNA, equal to only 1 % of the total, indicates an average lifetime of some 2–3 min. in *E. coli* growing with a generation time of about 1 hr.

Evidence for the turnover of the D-RNA, other than that given by the changing base composition of the newly formed RNA, appears from the delay in the entry of label into s-RNA and DNA. These polynucleotides seem to be synthesized to some extent from second-hand nucleotide material provided by the breakdown of the D-RNA. In chloramphenicol-treated cells, where D-RNA is apparently synthesized at the normal rate, but not degraded, this delay in labelling is eliminated.

Assuming an average molecular weight of 2×10^5, the number of D-RNA molecules thus estimated is equal to one-tenth the number of ribosomes. If this is all template RNA, then for a coding ratio of 3, each template molecule could be involved in the synthesis of as many as 75 protein molecules.

The Labile Nuclear Ribonucleic Acid of Animal Cells and its Relevance to the Messenger-Ribonucleic Acid Hypothesis

By H. Harris. (*Department of Cell Biology, John Innes Institute, Bayfordbury, Hertford, Herts.*)

The experiments were carried out on HeLa cells growing exponentially in suspension culture. The nuclei were isolated by the procedure of Fisher & Harris (1962) which permits the recovery of virtually all the nuclei from a population of cells. These nuclei contained between 98 and 99 % of the DNA of the cells and, within the limits of measurement, all the RNA in the nucleus labelled in a 10 min. exposure of the cells to a radioactive RNA precursor. The residual cytoplasm was separated by centrifugation into a microsomal and a supernatant fraction. The ribonucleic acids extracted from the nuclei and from the two cytoplasmic fractions were examined in the ultracentrifuge. If care was taken to minimize degradation, the rapidly labelled nuclear RNA sedimented as two components having sedimentation coefficients of approximately 28 s and 16 s. The microsomal RNA, when prepared in the same way, also had two components, with sedimentation coefficients similar to those of the nuclear RNA. The cytoplasmic supernatant fraction contained, apart from a major component

sedimenting at approximately 3 s, two minor components sedimenting at 28 s and 16 s. These minor components were not derived from the rapidly labelled nuclear RNA, but from the microsomal RNA.

The passage of radioactivity through these ribonucleic acids was studied in experiments in which the cells were first exposed for 10 min. to a radioactive RNA precursor and then incubated for 5 hr. in non-radioactive medium. The results indicated that the nuclear RNA which became labelled rapidly, and which had been shown in previous experiments (Watts & Harris, 1959; Harris, 1959; Harris & Watts, 1962) to be broken down within the cell, was not transferred to the microsomes in the cytoplasm before being broken down. The observations suggested that this labile nuclear RNA was broken down within the nucleus. This interpretation of the data was strengthened by the finding that the nucleus, but not the cytoplasm, contained an active enzyme which degraded the rapidly labelled nuclear RNA to nucleoside 5′-monophosphates; there is reason to believe that this RNA is also broken down to nucleoside 5′-monophosphates in the intact cell. The relevance of these findings to current theory about the mechanism by which information is transferred from nucleus to cytoplasm is discussed.

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COMMUNICATIONS

Stimulation of Respiration of Isolated Mitochondria by Manganese Ions

By J. B. Chappell, G. D. Greville and K. E. Bicknell. (*Department of Biochemistry, University of Cambridge, and Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

During studies of rat-liver mitochondria with the oxygen electrode it was observed that, in presence of added substrate and inorganic phosphate (P_i), addition of low concentrations of Mn^{2+} led to a burst of respiration. (A similar effect has been noted with Ca^{2+} by Chance, 1955.) The response was proportional to the Mn^{2+} concentration up to about 1 mM. The initial rate, but not the total amount, of the stimulated oxygen uptake increased with the quantity of mitochondria. The response was reduced on omission of substrate and, still more, of P and inhibited by antimycin or HCN. Amytal inhibited when substrates with NAD-linked dehydrogenases were used, but not with succinate. The respiratory phenomenon was accompanied by appearance of H^+ in the medium and uptake of Mn^{2+} and P_i by the mitochondria. The Mn^{2+} and P_i were not readily removed by washing the particles with salt solutions. The mitochondria accumulated up to 95% of the Mn^{2+} in the medium.

The H^+ production (at completion about 0·9 equiv. of H^+/atom of Mn^{2+} originally in the medium) was also sensitive to respiratory inhibitors. It was suppressed under anaerobic conditions and re-instituted by admission of oxygen. After the H^+ production was complete, addition of sufficient Triton X-100 to cause lysis returned the pH almost to the initial value. Low concentrations of Ca^{2+} (30 μM) had a similar effect to Mn^{2+}; with concentrations above 60 μM the initial H^+ production was followed by a spontaneous rise in pH, probably associated with increased mitochondrial permeability. With Mn^{2+}, P_i could be replaced by arsenate, but the H^+ production was not now reversed by Triton.

Uptake of Mn^{2+} and P_i and H^+ production thus appear to depend on respiration. Since inhibition was observed with dinitrophenol but not with oligomycin, some intermediate stage in the oxidative phosphorylation sequence may be involved.

No connexion between the phenomena described here and the action of Mn^{2+} in restoring oxidative phosphorylation in aged mitochondria or preventing swelling (see Ernster & Lindberg, 1958) is at present obvious. Bartley & Amoore (1958) also have observed Mn^{2+} uptake with H^+ production by liver mitochondria. Rothstein (1954) found metabolically dependent uptake of Mn^{2+} and P_i by intact yeast cells.

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Rate-Limiting Reactions in Mitochondrial Respiration

By J. B. CHAPPELL. (*Department of Biochemistry, University of Cambridge*)

Mitochondrial respiration may be stimulated by ADP together with phosphate or by agents which uncouple phosphorylation from respiration. It is widely held that the rates of ADP- and maximal 2,4-dinitrophenol- (DNP) stimulated respiration are equal. Investigation of the stimulated oxidation of various substrates by rat-liver and pigeon-heart mitochondria with the oxygen electrode has revealed that this equality rarely exists. The oxidizable substrates which have been used fall into two classes. With the first group, which includes succinate (in the presence of Amytal, to prevent malate oxidation) and malate (in the presence of a transaminating system), the rate of DNP-stimulated respiration is greater than the rate obtained with ADP. The second group of substrates, examples of which are α-oxoglutarate and β-hydroxybutyrate, show equality of ADP- and DNP-stimulated rates of oxidation. With this latter group it appears that the dehydrogenase systems are rate limiting, whereas with the former the rate of ADP-stimulated respiration is limited by the ability of the mitochondrion to synthesize ATP.

Thus, with succinate or malate (with transaminating system) or with a mixture of substrates of the second type a characteristic maximal rate of ATP synthesis has been observed. This was 0·34–0·39 μmole ATP/mg. of protein/min. for liver mitochondria at pH 7·2 and 25°; the corresponding value for heart sarcosomes was 0·95–1·2. These values corresponded closely to the maximum velocity of ATP hydrolysis induced by DNP, indicating that a common rate-limiting step controls both ATP synthesis and breakdown.

The relative activities of the respiratory chain and the dehydrogenase systems can control the course of mitochondrial oxidations. Thus with liver mitochondria, in which the activity of the succinate dehydrogenase exceeds that of the phosphorylation system, when ADP is used to stimulate respiration it was shown that malate and fumarate were the end-products of succinate oxidation. With pigeon-heart mitochondria, in which the activity of the ATP-synthesizing system exceeds that of the succinate dehydrogenase, the malate derived from succinate oxidation was also oxidized; this ultimately led to inhibition of succinate oxidation due to the accumulation of oxaloacetate. With both types of mitochondria low concentrations of DNP (about 10–20 μM) produced a one-step oxidation, whereas higher concentrations resulted in oxaloacetate production. Reducing the activity of the succinate dehydrogenase of liver mitochondria by adding low concentrations of malonate or reducing the activity of the phosphorylating system with sarcosomes by adding oligomycin led to behaviour which was consistent with the view that the relative activities of the dehydrogenase and respiratory chain determine the course of succinate oxidation.

It appears that competition for cytochrome *b* rather than the process of reversed electron transport is responsible for this phenomenon.

Soluble Adenosine Triphosphatase of Mitochondria

By M. J. SELWYN. (*Department of Biochemistry, University of Cambridge*)

Lardy & Wellman (1953) reported that the ATPase activity can be extracted from acetone-dried rat-liver mitochondria and following this the enzyme has been purified about one-hundredfold from aqueous extracts of acetone-dried beef-heart mitochondria. Two steps of ammonium sulphate fractionation followed by chromatography on diethylaminoethylcellulose are used. A similar purification scheme can be used for the ATPase of rat-liver mitochondria. Starch-gel electrophoresis shows only one band of ATPase activity after staining by the method of Wachstein & Meisel (1957).

The properties of this ATPase, including cold-lability, leave little doubt that it is the same enzyme as that prepared by Pullman, Penefsky, Datta & Racker (1960).

The enzyme is activated by the following divalent cations (in order of increasing activity): Ca, Ni, Cd, Mn, Zn, Co, Mg; this series shows a good correlation with the ionic radius. The trivalent lanthanide ions fail to activate the enzyme, probably because their complexes with ATP are insoluble, but they catalyse a rapid non-enzymic hydrolysis of ATP which shows a similar dependence on ionic radius.

Substituted phenols have been found to produce three types of effect but the type of effect is dependent not only on which phenol is present but also on which metal activator and substrate are being used. Type A effect, found with Co, Ni, or Mg as activator and ATP as substrate, is stimulation at low, but inhibition at high, phenol concentrations. This effect is shown for example by 2,4-dinitrophenol, pentachlorophenol, *p*-nitrophenol and picric acid. Type B effect is inhibition depending on the square of the phenol concentration and is shown by the phenols mentioned above when Mn, or Zn is the activator and ATP the substrate. It is also shown by 2,4-dibromophenol, 2,6-dinitrophenol

or *o*-nitrophenol acting on Mg- or Mn- activated ATPase. Type C effect which is simple inhibition has been found with ITP as substrate for every combination of phenol and metal activator that has been tried. The kinetics of phenol stimulation and inhibition suggest that the phenol is in equilibrium with enzyme–phenol complexes but that the substrate is not in equilibrium with enzyme–substrate complexes.

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Properties of Mitochondrial Adenosine Triphosphatase before and after Solubilization

By M. J. Selwyn and J. B. Chappell. (*Department of Biochemistry, University of Cambridge*)

The action of substituted phenols on isolated mitochondria results in uncoupling of the process of oxidation from phosphorylation, the appearance of ATPase activity and the inhibition of certain exchange reactions. The isolation and purification of a Mg-activated, cold-labile ATPase after mechanical disruption of beef-heart mitochondria (see Racker, 1961) or from aqueous extracts of acetone powders of sarcosomes or liver mitochondria (Selwyn, 1962) and the demonstration that this enzyme is stimulated by 2,4-dinitrophenol (DNP) (Lardy & Wellman, 1953) has led to the suggestion that this enzyme is the site of action of DNP in intact mitochondria.

From studies of intact mitochondria it is clear that oligomycin inhibits the terminal reaction of oxidative phosphorylation since it affects not only the overall process but also the DNP-stimulated ATPase activity and some exchange reactions. In contrast oligomycin does not inhibit the soluble ATPase from heart or liver mitochondria, nor does it affect the stimulation by DNP. The inhibition by oligomycin appears to depend on structural binding of the ATPase since this compound abolishes the Mg-activated ATPase of Keilin–Hartree preparations (Huijing & Slater, 1961) and markedly inhibits the same activity in Triton-treated liver mitochondria; in neither of these preparations does DNP cause stimulation. Aqueous extracts of acetone powder of Keilin–Hartree preparations yielded a Mg-activated, DNP-stimulated and oligomycin-insensitive ATPase which was also cold-labile, that is its properties were those of the enzyme prepared from intact mitochondria. Neither the soluble enzyme nor the ATPase of the Keilin–Hartree preparation was affected by arsenate, in contrast to the effect on the ATPase of intact mitochondria.

It seems that the isolated ATPase is normally bound to a component which suppresses DNP stimulation, prevents inactivation in the cold and enables oligomycin to act. This appears to be the case in the Keilin–Hartree preparation. In intact mitochondria this complex would appear to be associated with a further factor which couples it to the respiratory chain. This second factor would appear to be the site at which DNP acts in whole mitochondria. Support for this view derives from the effect of other phenols than DNP. For example, 2,4-dibromophenol and 2,6-dinitrophenol which cause rapid ATP hydrolysis with whole mitochondria fail to stimulate the soluble ATPase and indeed inhibit at high concentrations.

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The Stimulation by Acetyl-Coenzyme A of the Oxidation of Isocitrate

By W. C. Hülsmann and C. Benckhuijsen.* (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

Previous work (Hülsmann, 1960, 1962) pointed to the presence in heart sarcosomes of an enzyme catalysing reaction (1):

$$\text{oxalosuccinate} + \text{acetyl-CoA} \rightarrow \alpha\text{-oxoglutarate} + \text{malonyl-CoA} \quad (1)$$

The Keilin & Hartree (1947) heart-muscle preparation, made from horse or pig heart, contains isocitrate dehydrogenase (EC 1.1.1.42), NAD(P) transhydrogenase (EC 1.6.1.1) and the entire sarcosomal respiratory chain, except for the nicotinamide–adenine dinucleotides. The preparation catalyses the oxidation of isocitrate when NAD^+, $NADP^+$ and Mg^{2+} are added. The bivalent cation, which is necessary for the decarboxylation of the oxalosuccinate formed (Ochoa, 1948), can be replaced by

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catalytic amounts of acetyl-CoA, suggesting that reaction (1) followed by reaction (2) takes place:

$$\text{malonyl-CoA} \rightarrow \text{acetyl-CoA} + CO_2 \quad (2)$$

Malonyl-CoA can partially replace acetyl-CoA, and suboptimal amounts of the two thiol esters have a more than additive effect, suggesting that acetyl-CoA catalyses reaction (2), possibly by reactions (3) and (4):

$$\text{malonyl-CoA} + \text{acetyl-CoA} \rightarrow \text{acetoacetyl-CoA} + CO_2 + \text{CoA} \quad (3)$$

$$\text{acetoacetyl-CoA} + \text{CoA} \rightarrow 2\ \text{acetyl-CoA} \quad (4)$$

The maximum stimulation of isocitrate oxidation was obtained with 0·1 mM-acetyl-CoA. Since the rate of oxidation was not further stimulated by the addition of crude pig-heart isocitrate dehydrogenase, it is probable that the rate of NADPH oxidation is then the rate-limiting step.

The ability of acetyl-CoA to replace Mg^{2+} could also be demonstrated spectrophotometrically with the crude preparation of pig-heart isocitrate dehydrogenase. The initial rate of formation of NADPH in the presence of acetyl-CoA was proportional to the amount of enzyme preparation. The rate of reduction fell progressively with time, presumably due to removal of acetyl-CoA, since the further addition of the latter compound again stimulated the rate of reduction. This removal of acetyl-CoA required the simultaneous oxidation of isocitrate, since preincubation of the acetyl-CoA with the enzyme preparation alone did not remove its effectiveness in catalysing the oxidation of isocitrate. Preliminary experiments using hydroxylamine as trapping agent, followed by chromatography, suggest the intermediate formation of malonyl-CoA.

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The Role of High-Energy Intermediates in the Reduction of α-Oxoglutarate plus Ammonia by Malate in Rat-Liver Mitochondria

By J. M. Tager. (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

The synthesis of glutamate from α-oxoglutarate and NH_3 in rat-liver mitochondria can be coupled with the reduction of NAD^+ that occurs during the aerobic oxidation of succinate (Slater, Tager & Snoswell, 1962; Klingenberg & Haefen, 1962) or of tetramethyl-*p*-phenylenediamine in the presence of succinate and antimycin (Tager, Howland & Slater, 1962). Under these conditions, the malate derived from succinate gives rise to aspartate according to the reactions:

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+ \quad (1)$$

$$NADH + H^+ + \alpha\text{-oxoglutarate} + NH_3 \rightleftharpoons \text{glutamate} + NAD^+ \quad (2)$$

$$\text{glutamate} + \text{oxaloacetate} \rightleftharpoons \alpha\text{-oxoglutarate} + \text{aspartate} \quad (3)$$

Experiments were performed in which malate was used instead of succinate as donor of reducing equivalents for the synthesis of amino acids. When rat-liver mitochondria (4–9 mg. of protein) were incubated with KCl, $MgCl_2$, EDTA, tris–HCl, ADP (0·1 mM), inorganic phosphate, α-oxoglutarate, NH_3, malate, arsenite (to prevent the oxidation of α-oxoglutarate and of any pyruvate formed) and glutamate (to prevent the inhibition of malate oxidation by oxaloacetate), a net synthesis of glutamate and aspartate occurred. The synthesis was inhibited by antimycin or Amytal and either stimulated or not affected by oligomycin. The inhibition by antimycin or Amytal was overcome by adding ATP (10 mM). In the presence of antimycin or Amytal, the stimulation by ATP was prevented by oligomycin. In a typical experiment, Amytal decreased the amount of glutamate + aspartate synthesized in 20 min. from 7·2 to 1·6 μmoles. When ATP was added in addition to Amytal, 6·5 μmoles of glutamate + aspartate were formed, but if oligomycin was present as well as the Amytal and ATP, only 1·9 μmoles were found.

These and other results indicate that high-energy intermediates generated during malate oxidation catalyse the transfer of reducing equivalents from malate to α-oxoglutarate and NH_3 in rat-liver mitochondria. These high-energy intermediates can also be synthesized from ATP, but not if oligomycin is present (cf. Chance, 1962). This interpretation implies that malate dehydrogenase (EC 1.1.1.37) and glutamate dehydrogenase (EC 1.4.1.3) are located in different compartments of the mitochondrion (cf. Hemker, 1962) and differs in important respects from the mechanism proposed by Krebs (1961) for the reduction of NAD^+ coupled with the aerobic oxidation of succinate (Chance, 1956).

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On the Suggested Requirement of Adenosine Triphosphate for the Aerobic Oxidation of Succinate in Rat-Liver Mitochondria

By E. C. Slater and A. Kemp, Jun. (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

Azzone & Ernster (1961) have proposed that the aerobic oxidation of succinate by rat-liver mitochondria requires ATP. This proposal is based on their finding that preincubation of the mitochondria suspended in a phosphate-free sucrose–KCl medium with 2 mM-arsenate for 3–4 min. results in a marked depression of the succinate-oxidase activity, measured after addition of 2,4-dinitrophenol or dicoumarol, and that addition of ATP after the preincubation restores the oxidation. It was shown that the preincubation resulted in the loss of phosphate compounds from the mitochondria.

This interpretation has been challenged by Chappell (1960) and others, who have suggested that the inhibition is due to the formation of oxaloacetate during the preincubation, and that the oxaloacetate is removed by ATP. Azzone & Ernster (1961) have, however, reported that the amount of oxaloacetate present in their mitochondria after the incubation was less, on a molar basis, than the amount of succinate dehydrogenase (see also Schollmeyer & Klingenberg, 1961).

Our studies have brought forward the following evidence against the interpretation of Azzone & Ernster (1961): (1) Restoration by ATP of the oxidation of succinate occurs only after a definite time lag; the oxidation then continues at a uniform rate, even when the added ATP is removed by the addition of hexokinase and glucose. (2) The inactivation is not specific for succinate oxidation, since the rate of oxidation of glutamate, pyruvate + malate, α-ketoglutarate or β-hydroxybutyrate is also greatly decreased. In these cases, little if any reactivation is obtained with ATP. (3) The reactivation of succinate oxidation is not specific for ATP, since serum albumin is also active (cf. Schollmeyer & Klingenberg, 1961), although rather less effective than ATP. (4) As Chappell (1960) has emphasized, the reactivation by ATP of succinate oxidation is not sensitive to oligomycin, which blocks the interaction of ATP with the respiratory chain (Lardy, Johnson & McMurray, 1958).

It is possible that pre-incubation with arsenate, with consequent loss of high-energy compounds, causes structural damage to the mitochondria (swelling can be demonstrated by the light-scattering method), perhaps followed by formation of inhibitory unsaturated fatty acids, and that ATP or serum albumin is able partially to reverse these effects, so that the mitochondria are able to oxidize succinate.

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Spectral Evidence for Enzyme–Inhibitor Complexes of Succinate Dehydrogenase

By D. V. Dervartanian and C. Veeger (introduced by E. C. Slater). (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

The addition of succinate to soluble succinate dehydrogenase causes a decrease in absorption of about 18 % in the flavin region (Singer, Kearney & Bernath, 1956; Wang, Tsou & Wang, 1956). Addition of dithionite gives a still further decrease in absorption which can be restored to the level obtained with succinate by addition of fumarate (Massey & Singer, 1957).

When fumarate, in the absence of reducing agent, is added to the preparation of Wang *et al.* (1956), a decrease in absorption is observed between 400 and 470 mμ and an increase between 480 and 540 mμ, with a maximum at 510 mμ in the difference spectrum.

Three types of response were obtained with other competitive inhibitors. (1) Maleate (K_i, 6·0 mM), itaconate (K_i, 1·8 mM) and malonate [K_i, 45 μM (Keilin & King, 1960)] gave similar changes in spectrum to those obtained with fumarate. This has previously been reported for malonate by Kearney (1957). (2) Malate (K_i, 35 mM) and oxaloacetate [K_i, 1·5 μM (Pardee & Potter, 1948)] caused a greater decrease between 400 and 480 mμ, with a minimum in the difference spectrum at 460 mμ instead of at 450 mμ, while the 480–540 mμ band is replaced by a wider band between 500 and 700 mμ, with maximum at 575 mμ (malate) or 600 mμ

(oxaloacetate). (3) Pyrophosphate [K_i, 0·23 mM (Keilin & King, 1960)], which unlike the other competitive inhibitors is not obviously structurally related to succinate, had no effect on the spectrum.

Compounds such as pyruvate (K_i, 0·13 M), crotonate and α-ketoglutarate, which are fairly closely related structurally to succinate but which have little or no inhibitory action, have no action on the spectrum.

Since the enzyme preparation of Wang *et al.* (1956) is already activated, we do not interpret these changes in terms of activation of the enzyme, as has been done by Kearney (1957) for malonate, but in terms of enzyme–inhibitor complexes of the type proposed for fumarate by Slater (1955). The nature of these enzyme–inhibitor complexes could be attributed to charge-transfer phenomena of the flavin with the different inhibitors. However, it may be significant that the most pronounced spectral changes are found with malate and oxaloacetate, which contain a methylene group in the same relation to the two carboxyl groups as in succinate. This suggests the possibility that the spectrum is that of a flavin semiquinone.

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Effect of 3-Hydroxyanthranilic Acid on the Cellular Respiration at Mitochondrial Level

By E. Quagliariello, S. Papa and C. Saccone (introduced by Committee). (*Department of Biochemistry, University of Bari, Italy*)

3-Hydroxyanthranilic acid, an intermediate in the tryptophan–nicotinic acid interconversion, appears in considerable quantities in the urine of patients with cancer of the bladder (Boyland & Williams, 1955; Quagliariello, Auricchio, Casale & Tancredi, 1958; Tompsett, 1959); besides, Musajo, Benassi & Parpajola (1956) have found this compound in the urine of leukaemia cases. In 1956, Boyland & Watson showed that the 3-hydroxyanthranilic acid can produce cancer of the bladder, and Ehrhart & Georgii (1959) have found that this acid is capable of inducing certain forms of leukaemia. The purpose of this paper is to present the results of our studies (see also Quagliariello, Papa & Saccone, 1961, 1962) on the effect of 3-hydroxyanthranilic acid on cellular respiration, on the oxidation of α-oxoglutarate, DL-β-hydroxybutyrate and succinate in rat-liver mitochondria, and on the phosphorylation coupled to these oxidations. The mitochondrial fraction was prepared by our modification (Quagliariello *et al.* 1962) of the method of Schneider & Hogeboom (1957). The yield was about 20 mg. of mitochondrial protein/g. of liver. The purity of mitochondria was shown by the P/O ratios, which were almost exactly equal to the theoretical values. Manometric assays were carried out according to the conventional Warburg technique. Inorganic phosphate was determined by the method of Lowry & Lopez (1946) and protein by the method of Cleland & Slater (1953).

From the experiments it appears that 3-hydroxyanthranilic acid (at 0·25–1 mM) inhibits the oxidation of oxoglutarate. This inhibition seems to be specific for oxidative decarboxylation of oxoglutarate: in fact there is no effect on the oxidation of hydroxybutyrate and succinate. Furthermore, the inhibition becomes more marked when, by adding malonate to the system, the oxidation is almost completely limited to the first stage of oxidative decarboxylation. Moreover, the 3-hydroxyanthranilic acid uncouples the phosphorylation linked to ketoglutarate and hydroxybutyrate oxidation (in the last case it lowers the P/O ratio by exactly one unit). Using succinate as respiratory substrate, one observes only a very slight uncoupling effect which we consider to be due to the effect of 3-hydroxyanthranilic acid on the phosphorylation linked to the further oxidation of the malate which is produced in the process. From these results it seems that 3-hydroxyanthranilic acid only acts on the first of the three steps of oxidative phosphorylation: the phosphorylation coupled to oxidation of $NADH_2$.

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Effects of Salicylate on the Metabolism of [1,4-$^{14}C_2$]Succinate by Rat-Liver Mitochondria

By C. Bryant and M. J. H. Smith. (*Empire Rheumatism Council Research Unit, Chemical Pathology Department, King's College Hospital Medical School, London, S.E.* 5)

Suspensions of rat-liver mitochondria were found to incorporate radioactivity from [1,4-$^{14}C_2$]succinate into fumarate, malate, aspartate, alanine, citrate and glutamate. In the presence of 5 mM-salicylate there was an increased accumulation of the isotope into fumarate and malate. After disruption by ultrasonic vibrations the mitochondrial preparations incorporated ^{14}C from the labelled succinate into fumarate, malate and aspartate but not into citrate and glutamate, suggesting that the radiocarbon was not transferred beyond oxaloacetate. Salicylate caused an increased incorporation of radioactivity into fumarate and malate but reduced the formation of radioactive aspartate.

One possible explanation of these results is that salicylate interfered with the conversion of oxaloacetate into malate and we have found that salicylate inhibits pig-heart malic-dehydrogenase activity *in vitro*. The effects of chemically related compounds on the enzyme activity and on intermediary metabolism in mitochondria were also discussed.

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Incorporation *in vitro* of Amino Acids by Nucleoli and Subnuclear Fractions of Rat Liver

By G. F. Rowland, K. R. Rees and J. S. Varcoe. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

Rat-liver nuclei isolated in 0·25 M-sucrose as described by Rees & Rowland (1961) were shown to retain their ability to incorporate [^{14}C]glycine into protein after sonic disruption (Rees, Ross & Rowland, 1961). The largest components of the 'sonicate' appeared to be nucleoli and these were isolated by centrifugation at 2100*g* for 20 min. The remainder of the sonicate was separated into four particulate fractions (B to E) and a soluble fraction (F) by four successive centrifugations ($2·5 \times 10^5$, $1·2 \times 10^6$, $6·3 \times 10^6$ and $3·2 \times 10^7 g$ av.min).

Each fraction was resuspended in 0·25 M-sucrose and incubated at 37° for up to 3 hr. with [^{14}C]-glycine, [^{14}C]valine or [^{14}C]leucine in a medium containing only phosphate buffer and sodium chloride. On determination of radioactivity of the protein, the nucleoli and fractions D and E were found to have a much higher specific activity than intact nuclei whereas the other fractions showed negligible incorporation.

Chemical analysis showed that the nucleoli have a high RNA to protein ratio, that B and C, which comprise the bulk of the sonicate, are rich in DNA, that D and E are rich in phospholipids but contain very little nucleic acids and that F is mainly soluble protein.

It was observed that increased sonication time transferred the ability to incorporate amino acids from the nucleolus to fractions D and E suggesting that an active particle associated with the nucleolus is liberated by sonication and sedimented with D and E. Chemical analysis indicates that this material may be the heterochromatin surrounding the nucleolus.

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Metabolism of Rat-Liver Nuclei

By A. K. Huggins* and G. F. Rowland. (*Empire Rheumatism Council Research Unit, King's College Hospital Medical School, London, S.E.* 5 *and Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

The ability of rat-liver nuclei, isolated in a sucrose–calcium medium (Rees & Rowland, 1961), to utilize various externally supplied substrates has been investigated. The incorporation of radioactivity from [^{14}C]glucose, [1-^{14}C]valine, [1-^{14}C]-leucine, [2-^{14}C]acetate or [1,4-$^{14}C_2$]succinate into the soluble intermediates formed by the nuclear preparations incubated at 37° for periods of up to 60 min. was studied by two-dimensional paper-chromatography followed by radioautography (Smith & Moses, 1960).

^{14}C-Labelled soluble intermediates were not detected except when labelled succinate was used. Over 90 % of the radioactivity originally present in the succinic acid was incorporated into fumaric, malic and aspartic acids in 30 min. The distribution of ^{14}C differs qualitatively from that obtained

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previously with rat-liver mitochondria under similar conditions (Huggins, Bryant & Smith, 1961) in that no radioactivity was present in citric acid, glutamic acid or alanine. Comparative studies of the utilization of succinate by mitochondria and cytological investigations of the nuclear preparations have been made to determine if the observed metabolism of succinic acid can be accounted for by contamination with mitochondrial material.

We wish to thank the Empire Rheumatism Council and the British Empire Cancer Campaign for grants towards the cost of this work.

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The Oxidation of Cytochrome *c* by Horse-Radish Peroxidase

By P. Nicholls. (*Molteno Institute, University of Cambridge*)

The oxidation of cytochrome *c* by hydrogen peroxide catalysed by horse-radish peroxidase (HRP) has been followed spectrophotometrically. The kinetic behaviour differs from that found in the oxidation catalysed by yeast cytochrome *c* peroxidase (CcP) and that shown by mammalian cytochrome oxidase (Beetlestone, 1960; Smith & Conrad, 1956). Thus in the HRP system neither ferric cytochrome *c* nor salmine is inhibitory. And although in dilute buffer the time course of the HRP-catalysed oxidation is complicated, in 2 M-KCl the reaction is strictly zero order (cf. Chance, 1951) down to 2 μM-cytochrome *c*. Variation of peroxide concentration does not affect the rate constant (8 sec.$^{-1}$; pH 7, 20°) which can be attributed to some intramolecular reaction. This reaction cannot involve a cytochrome *c*–enzyme complex, as is possible for the CcP- and oxidase-catalysed oxidations (Nicholls, 1961; Minnaert, 1961).

If the usual bimolecular process by which ferrocyanide is oxidized by HRP is suppressed, using a medium of high ionic strength, a similar zero-order reaction is found ($k \simeq 5$ sec.$^{-1}$). This oxidation of ferrocyanide cannot be directly correlated with a reaction of either peroxidase–peroxide compound; as in the oxidation of cytochrome *c* by CcP or HRP (Chance, 1950), it may be prevented by preincubation of HRP with peroxide.

These phenomena suggest that the limiting process may be the oxidation of part of the 'endogenous donor' (BH), as with ascorbic acid (Chance, 1949). The oxidation of cytochrome *c* can then occur by two pathways, as indicated in equation (1):

$$\mathrm{HRP(BH)} \xrightarrow[\text{fast}]{\mathrm{H_2O_2}} \text{Comp. I} \xrightarrow[\text{fast}]{\text{cyt. } c} \text{Comp. II} \begin{cases} \xrightarrow{\text{cyt. } c} \mathrm{HRP(BH)} \\ \mathrm{HRP(B)} \xrightarrow{\text{cyt. } c} \mathrm{HRP(BH)} \end{cases} \quad (1)$$

The catalysis by CcP (Beetlestone, 1960) may involve an initial complex formation between cytochrome *c* and the enzyme. A similar mechanism can be given for the more complicated case of cytochrome oxidase.

The present evidence supports the general hypothesis that enzymic oxidation of cytochrome *c* proceeds by intramolecular electron transfer (Nicholls, 1961), and suggests that the peculiarities of such oxidations may be resolved if non-haem redox groups occur on the enzymes concerned, as postulated by Lemberg & Legge (1949).

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Some Reactions of Cytochrome Oxidase

By Q. H. Gibson and C. Greenwood. (*Department of Biochemistry, University of Sheffield*)

Gibson, Greenwood & Massey (1960) reported measurements of the rate of reaction with O_2 of cytochrome-oxidase preparations made with snake venom and with cholate. The observations have now been extended to the preparation of Yonetani (1960, 1961) which is stabilized with Emasol 4130. The reaction with low concentrations ($< 3\ \mu$M) of oxygen gives an apparent second-order constant of about 6×10^7 M^{-1} sec.$^{-1}$ at pH 7·4, 20°. The rate is higher when measured at 605 mμ than at 445 mμ, and increases as the reaction proceeds. At higher oxygen concentrations (20–500 μM) the reaction is biphasic; a rapid stage dependent on oxygen concentration is followed by a slow step ($\approx$ 700 sec.$^{-1}$) which is independent of oxygen concentration. At 445 mμ the rapid phase accounts for about half the total extinction change; at 410 and 605 mμ the proportion of rapid change is much less. These results fit well with Yonetani's (1960) spectra for

cytochromes a and a_3 if the rapid phase of the extinction change is assigned to the reaction of oxygen with a_3, which in turn oxidizes a. Whereas the rapid reaction of a_3 with oxygen is a stable property of the enzyme, the oxidation of a is labile, and may become very slow indeed ($t_{50} \approx 1$ sec.) after drastic treatment.

The Yonetani preparation combines with CO with a rate constant of 1×10^5 M^{-1} sec.$^{-1}$ at pH 7·4, 20°) and an activation energy of 6·4 kcal. The rate of dissociation of CO has been measured by displacement with NO and is 0·023 sec.$^{-1}$ under the same conditions. Although the initial rate of displacement of CO from CO–cytochrome oxidase by O_2 is the same as is found with NO, the rate increases rapidly to reach 8–10 times its initial value.

The combination of cyanide with reduced cytochrome oxidase gives rather small extinction changes (Yonetani, 1960), but the rate constants for both combination and dissociation reactions can be obtained by displacement with CO and by competitive combination experiments. The rates are $1{\cdot}2 \times 10^2$ M^{-1} sec.$^{-1}$ and 0·08 sec.$^{-1}$. It appears that cyanide and CO combine with the same group in the enzyme.

Titration with CO and with O_2 shows that an amount of enzyme which can bind 1 CO donates 6 electrons on oxidation. As cytochrome oxidase appears to contain Fe and Cu in a 1:1 ratio we suggest that the functional unit contains $2a_3$, $4a$ and 6 Cu. The a_3:a ratio agrees with the proposals of Ehrenberg & Yonetani (1961) based on determinations of magnetic susceptibility.

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The Mechanism of Liver Alcohol Dehydrogenase

By K. Dalziel.* (*Department of Biochemistry, University of Sheffield, Sheffield* 10)

Initial-rate data for liver alcohol dehydrogenase in neutral and acid solution are inconsistent with some of the requirements of compulsory order mechanisms, including the Theorell–Chance mechanism (Dalziel, 1957; Dalziel & Theorell, 1957; Dalziel, 1962a). First, the rate of dissociation of the enzyme–NAD complex calculated from kinetic parameters ($\phi_{12}/\phi_1\phi_2$) for the alcohol–NAD reaction is smaller than the maximum rate of the reverse reaction, by a factor of 0·3 at pH 6·0 and 0·5 at pH 7·1. Secondly, kinetic values for the dissociation constant of the enzyme–NAD complex (ϕ_{12}/ϕ_2) are smaller, by the same factors, than independent values (Theorell & McKinley-McKee, 1961). These discrepancies seemed to be clearly outside the random error of initial-rate measurements (Dalziel, 1962a).

For a compulsory-order mechanism, the initial-rate equation in presence of a molar ratio, r, of competitive inhibitor to coenzyme, S_1, is

$$\frac{e}{v_0} = \phi_0\left(1+\frac{K_{m_1}r}{K_i}\right)+\frac{\phi_1}{[S_1]}+\frac{\phi_2}{[S_2]}\left(1+\frac{K_{s_1}r}{K_i}\right)+\frac{\phi_{12}}{[S_1][S_2]}.$$

This relation explains the large effects of small amounts of competitive inhibitor in $NADH_2$ preparations on the maximum rate ($1/\phi_0$) of aldehyde reduction by liver alcohol dehydrogenase (Dalziel, 1961, 1962b, c), since at pH 6·0 the Michaelis constant for $NADH_2$, K_{m_1}, is much larger than its substrate constant, K_{s_1}. The opposite is true in the reverse reaction: for NAD at pH 6·0, $K_{m_1} = 4\,\mu$M and $K_{s_1} = 266\,\mu$M. The above equation shows that the presence in an NAD preparation of only 3% of adenosine diphosphate ribose, $K_i = 7\,\mu$M (Dalziel, 1962d), or of 0·1% of $NADH_2$, $K_i = 0{\cdot}23\,\mu$M, for example, would give an apparent value for ϕ_2 twice the true value, but ϕ_0 would not be significantly affected.

Commercial NAD of high purity was further purified by a modification of the method of Wallenfels & Christian (1957). In initial-rate measurements at pH 6·0 with ethanol as substrate, the product gave a value of ϕ_2 smaller by a factor of 0·35 than that obtained with the original coenzyme preparation, whilst ϕ_0 was unchanged.

These findings indicate that the kinetic discrepancies from the requirements of a compulsory-order mechanism are due to small amounts of an inhibitor in NAD preparations, and that within the experimental error the Theorell–Chance mechanism is valid for liver alcohol dehydrogenase. The remarkable sensitivity of initial-rate parameters to impurities in coenzyme preparations is presumably not confined to this enzyme.

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The Assay and Coenzyme-Binding Sites of Liver Alcohol Dehydrogenase

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Crystalline liver alcohol dehydrogenase (LADH) was prepared and then assayed by activity and binding-site concentration measurements.

(1) e_A (the activity enzyme concentration) = $1{\cdot}13/v \times t_{0{\cdot}2} \times 84\,000$ moles/l. (Dalziel, 1958), where 84 000 is the molecular weight of LADH and 1·13 depends on the mg. of LADH protein/ml., E_{280} (1 mg. of protein/ml.) being considered to be 0·42.

(2) e_B (binding-sites concentration) is obtained from a spectrophotofluorimetric titration (Theorell & Winer, 1960), and depends on the very low dissociation ($K = 5{\cdot}5 \times 10^{-9}$ M) and high fluorescence (Q = 38–40) of the LADH–IB–β-$NADH_2$ complex formed on titrating LADH + excess of isobutyramide (IB) with $NADH_2$ (Theorell & McKinley-McKee, 1961). In conjunction with e_A, e_B determines the integral number of binding sites/molecule. Similar titrations at other pH, when compared with those at pH 7, provide an extremely sensitive method of detecting any change in the number of binding sites/molecule.

It was found that $e_A \neq e_B$; the ratio being $0{\cdot}83 \pm 0{\cdot}01$. Previously e_B had been stated to be 10–20% lower than e_A (Theorell & Winer, 1960). e_B is considered accurate and 17% less than e_A due to, either (1) low molecular weight impurities resulting in a low value being used for the molecular weight, and/or for E_{280} (mg. of LADH protein/ml.), or (2) if the protein is pure, to an inaccurately determined value for the extinction being used.

For $e_A = e_B$ and the Dalziel assay to result in the correct enzyme concentration, $e_A = 1{\cdot}13/v \times t_{0{\cdot}2} \times 101\,000$ or $e_A = 0{\cdot}94/v \times t_{0{\cdot}2} \times 84\,000$ moles/l. must be used. With 101 000 (84 000/0·83) the error is considered to be in the molecular weight alone, while 0·94 implies a E_{280} (1 mg./ml.) = 0·505 (0·42/0·83). Except for the limits 84 000–101 000 and 0·42–0·505 the molecular weight and extinction/g. of LADH are at present uncertain. Prior to the value 84 000 (Ehrenberg & Dalziel, 1958) the molecular weight was 73 000 (Bonnichsen & Brink, 1957), the latter value being attributed to low-molecular-weight impurities.

The determination of e_B confirmed that, at pH 7, 2 $NADH_2$ molecules attach/LADH molecule and that this was invariant from pH 6 to 10 and most probably to pH 11. There was no indication of an abrupt change to 1 $NADH_2$/LADH at pH 10 as has been suggested from spectrophotometric (Bonnichsen & Brink, 1957) and more recently from optical rotatory dispersion (Li, Ulmer & Vallee, 1962) titrations. Inspection of the spectrophotometric titration indicates considerable uncertainty in the equivalence point, the position of which could also be due to the presence of glycine which forms an LADH–glycine complex (Theorell & McKinley-McKee, 1961). The divergent results could be compatible if IB, which complexes strongly, stabilizes the coenzyme on the second site. However, this seems somewhat unlikely as the two sites, which each contain one zinc atom, generally are independent and equal. Full details are being published.

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The Mechanism of Action and the Binding Sites of Liver Alcohol Dehydrogenase

By J. S. McKinley-McKee. (*Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

Liver alcohol dehydrogenase (LADH) acts by forming binary and ternary complexes with coenzymes and substrates (Theorell & McKinley-McKee, 1961*a–d*). Previously there were indications that at lower pH the interconversion of the ternary complexes affected the rate (Dalziel & Theorell, 1957; Theorell & Winer, 1959). However, spectrophotofluorimetric equilibrium (pH 6–10) and kinetic (pH 7 and 9) measurements showed that kinetically the Theorell–Chance mechanism applies (Theorell & Chance, 1951; Theorell & McKinley-McKee, 1961*a–d*).

It was possible to determine the dissociation constants of the various complexes formed, as the fluorescence and catalytic sites were found to be identical. The stability of LADH–$NADH_2$ increased rapidly at higher pH suggesting that SH groups join the protein to the 6-position of the pyridine ring. The increasing stability of LADH–NAD with increasing pH indicated that the ionization of a water molecule attached to zinc held together the

positive zinc and pyridinium centres. The imidazole complexes were interesting as imidazole competed with both aldehyde and alcohol. It also forms complexes with free zinc ions, which suggested that the LADH zinc attached the substrates. Many lines of evidence suggested that the coenzymes attached as bidentate chelates. Zinc is indeed the centre of the binary and ternary complexes (Theorell & McKinley-McKee, 1961*c*), the structure of which depends on the fact that stable zinc complexes are either tetrahedral or octahedral. The complexes incorporating monodentates appear to be tetrahedral with zinc joined to the protein by three bonds, the fourth to the monodentate; this being water in the free enzyme. Bidentate-containing complexes (e.g. with glycine, 1, 10-phenanthroline, the coenzymes) necessitate the octahedral configuration. In the coenzyme complexes the extra two chelate bonds are from the 6, 7- or 1, 6-positions of adenine.

Proof that there are other binding sites is seen from the stereospecificity of the reaction with regard to the coenzymes and substrates (Vennesland, 1957). The coenzyme ring may be orientated by the amide group hydrogen bonding to the enzyme (amide–zinc bonds are not possible). Whether the effect of the amide group is due to hydrogen bonding or is an electrostatic effect on the ring is uncertain. However, amide hydrogen bonding is not essential for the coenzyme ring to receive stereospecific rigidity, as protein SH addition to the pyridine ring or the N^+–OH^-–Zn bonding with the pyridinium ring could suffice. The substrates appear joined to zinc through their polar oxygen, while their conformation also seems determined by secondary attachments to the enzyme through their alkyl groups (Vennesland, 1957).

Competition experiments with submaximal concentrations indicate that each coenzyme when attached to the enzyme affects the monodentate bond differently, so determining its bond type and the ligand attaching. Thus the negative alcoholate ion can only attach with the assistance of the positive pyridinium ring and in this way unreactive complexes like LADH–NAD–aldehyde are prevented. Experiments carried out using fluoroethanol, fluorescent alcohols and isotopically labelled substrates also bear on the above.

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Contribution to the Determination of the Rennin-Sensitive Linkage in κ-Casein

By P. Jollès, C. Alais and Jacqueline Jollès. (*Laboratory of Biological Chemistry, Faculty of Science. University of Paris, Paris, France and Department of Microbiology, Jouy-en-Josas, France*)

When rennin is allowed to react with κ-casein from cow milk at pH 6·8 para-κ-casein is precipitated and the supernatant contains a caseino-glycopeptide which does not dialyse and is soluble in 12% trichloroacetic acid (TCA). Carboxypeptidase liberates Val, Thr, Ala and Ser from κ-casein, the same amino acids from the caseino-glycopeptide and Phe and Leu from para-κ-casein, but it has not been determined if there are one or more peptide chains (Jollès, Alais & Jollès, 1962).

κ-Casein has been submitted to reduction with $LiBH_4$ in aqueous medium at pH 8. After destruction of the excess of reagent, the solution is dialysed against water at 2°. A precipitate (A) is obtained and the supernatant contains a substance (B) which does not dialyse and is soluble in 12% TCA.

After total hydrolysis, (A) and (B) have been analysed. (A) seems to be almost identical to para-κ-casein. A study has also been undertaken to determine if some amino alcohols have been formed. Phenylalaninol has been detected. (B) seems similar to the reduced caseino-glycopeptide, but has not been studied.

From these preliminary experiments it can be concluded that $LiBH_4$ reduces the rennin-sensitive linkage in κ-casein, which seems to be an ester linkage involving the *C*-terminal Phe residue of para-κ-casein. It has not been determined whether this amino alcohol is the only one formed. $LiBH_4$, contrary to rennin, splits also some linkages in the caseino-glycopeptide part of κ-casein, as natural and reduced caseino-glycopeptides are not similar.

Jollès, P., Alais, C. & Jollès, J. (1962). *Arch. Biochem. Biophys.* (in the Press).

Variation of Substrate Concentration with $V_{max.}$ in Crude Tissue Extracts

By R. A. Dale.* (*Department of Chemical Pathology, Postgraduate Medical School of London, London W. 12*)

If it is accepted that in cells, in the resting steady state, many enzymes are unsaturated with respect to substrate (Racker, 1954; Krebs, 1959; Webb, 1961), and, furthermore, that the remainder of the system between the sites of formation and transformation of substrate is unsaturated also, then it may be predicted, with certain provisos, that the amount of substrate will vary directly with the amount of enzyme. The argument is as follows.

The amount of a substrate present in such a system is determined by the rate of release from the site of formation, that is, by the flux from the preceding enzyme in the sequence, by the volume between the active centres of the enzymes preceding and succeeding the substrate, and finally by the amount and K_m of the enzyme under consideration, that is, of the enzyme succeeding the substrate.

If in a similar system the amounts of the enzymes, and of the resting steady-state flux of substrate were larger, then the resting levels of substrate would be larger also.

The results of an attempt to demonstrate whether such a relationship exists for aldolase and fructose diphosphate and for lactate dehydrogenase and pyruvate in crude extracts of several rat tissues are presented in this paper. The amounts of the enzymes are represented by their $V_{max.}$. It is accepted that resting steady-state levels of fructose diphosphate and pyruvate in rat tissues can be measured (Dale, 1962). The tissues used include erythrocytes, kidney, liver, muscle, sarcoma and testis.

The relationship of the values obtained for the $V_{max.}$ of aldolase and the concentration of fructose diphosphate over all of the tissues examined is linear, the values for erythrocytes being near the origin and those for muscle being at the distal end of the curve. No such relationship was obtained for lactate dehydrogenase and pyruvate.

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Studies on the Active Centre of Yeast Hexokinase

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Active centre studies on hexokinase have been commenced by defining the presence of and requirement for –SH groups, a subject of conflicting reports earlier (Bailey & Webb, 1948; Berger, Slein, Colowick & Cori, 1946). Enzyme of high specific activity, separated in physically homogeneous state by chromatography on DEAE-cellulose (cf. also Trayser & Colowick, 1961), was employed here.

No –SH groups were detectable by CH_3HgNO_3 (Edelhoch, Katchalski, Maybury, Hughes & Edsall, 1953) or by *p*-chloromercuribenzoate (Boyer, 1954) titrations in water at 25°, but 3·4–4·1 –SH per molecule (mol.wt. 47 000) were titrated with CH_3HgNO_3 in 5·4 M-guanidine at 2°. Four cysteine residues per molecule are present, by an amino acid analysis (Spackman, Stein & Moore, 1958) kindly performed by Dr A. Tsugita and to be presented elsewhere (Ramel & Schachman, in preparation). It has previously been shown (Ramel, Stellwagen & Schachman, 1961) that the 47 000 molecule can be split by detergent into 2 inactive sub-units.

No effect on the activity at 25° was exerted by cysteine (0·02 M), nor by a 50-fold molar excess of CH_3HgNO_3 or *p*-chloromercuribenzoate or $HgCl_2$. However, at 35° (pH 8·0) CH_3HgNO_3 (5×10^{-5} M) abolished the activity. This loss was reversed by cysteine (0·01 M).

Sodium bromoacetate (0·18 M, pH 7) irreversibly inactivated hexokinase, but at appreciable rates only above about 30°, the rate increasing abnormally sharply over the range 30–37°. The inactivation rate, at 35°, increased sharply from pH 7 ($t_{\frac{1}{2}}$ = 31 min.) to pH 8 ($t_{\frac{1}{2}}$ = 3 min.), with only a slight pH dependence below 7. The rates of the bromoacetate and CH_3HgNO_3 inactivations were similar (at pH 8·0, 35°).

Isolation of the protein after complete inactivation (pH 8·0, 35°) by [^{14}C]bromoacetate showed 3·9 (± about 0·4) carboxymethyl groups per molecule. Glucose (0·01 M) protected considerably from bromoacetate inactivation.

The protein incubated alone (0·18 M-acetate) showed at 30–37° a very much slower activity loss which, however, showed the same general dependence upon pH and temperature. It was not reversed by cysteine (0·01 M).

The results suggest that 1 to 4 –SH per molecule (mol.wt. 47 000) are required for the active centre(s). These groups are not normally available to the reagents but become so after, probably, a time-dependent structural change occurring above

30°. Further study will be required to define the reaction site more precisely.

This work was performed at the Virus Laboratory, University of California, Berkeley (U.S.A.), during the tenure (E.A.B.) of a Rockefeller Fellowship, 1960–61, while on leave of absence from King's College, London, and of (A.R.) a U.S. Public Health Service Fellowship. The authors acknowledge with gratitude the facilities made available there by Dr H. Fraenkel-Conrat and Dr H. K. Schachman.

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The Constituent of Wheat Binding Latent β-Amylase

By E. V. Rowsell and L. J. Goad. (*Department of Biochemistry, Manchester College of Science and Technology, Manchester* 1)

About 80% of the β-amylase of ungerminated wheat kernels occurs as an inactive latent form. The latent enzyme has been assayed by observing the increase in β-amylase activity after treating wheat with papain. The latent is converted into the active form on germination (Geddes, 1946). It has been suggested that inactive β-amylase in cereals is so because it is bound to protein; that it is released when the protein is degraded (Myrbäck & Neumüller, 1950). The object in the present work has been to discover the constituent of wheat with which latent β-amylase is associated.

Methods of extracting albumins, globulins and gliadin from wheat flour (endosperm) were those used by Bilinski & McConnell (1958). β-Amylase was assayed measuring reducing sugar liberation from starch (Bernfeld, 1955), and was characterized by observing limit-dextrin formation. We have found all the active β-amylase in the albumin–globulin fraction. Activity in this fraction was increased by about 50% with the addition of cysteine. The gliadin contained neither free nor bound enzyme. The insoluble residue (glutenin plus starch) had no β-amylase action but the enzyme was released from it into solution by papain activated with cysteine. More than 80% of the latent β-amylase of flour has been recovered in this fraction. Mechanical disintegration with ultrasonic vibration, homogenization, or repeated freezing and thawing were not effective in releasing the bound enzyme; nor did these treatments denature it. The latent enzyme remained bound after extraction of glutenin–starch or flour with butanol for 1 hr. or after hot Soxhlet-extraction with ether for 24 hr.

The lighter starch particles were separated from glutenin–starch by repeating a sequence of suspension in water, low-speed centrifugation and decantation. Bound β-amylase was found only in the glutenin fraction. Using salivary α-amylase virtually all the starch has been digested away with little loss of bound enzyme from the remaining insoluble glutenin. No β-amylase could be extracted from undamaged starch granules from whole wheat. Evidently none is bound to the protein which adheres to intact starch granules (Hess, 1954).

Apparently the latent β-amylase of wheat is chemically bound to glutenin.

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Latent β-Amylase of Wheat: Its Mode of Attachment to Glutenin and its Release

By E. V. Rowsell and L. J. Goad. (*Department of Biochemistry, Manchester College of Science and Technology, Manchester* 1)

Papain and sulphydryl reagents increase β-amylase activity in ungerminated cereals. The suggestion has been made that sulphydryl reagents act indirectly by activating cereal proteases; that proteolytic action is necessary for the release of latent β-amylase (Myrbäck & Örtenblad, 1938; Geddes, 1946; Myrbäck & Neumüller, 1950).

We have investigated the mode of release of soluble active β-amylase from its inactive glutenin-bound form (Rowsell & Goad, 1962). β-Amylase was brought into solution when wheat glutenin–starch in aqueous suspension was extracted with papain activated by cysteine, trypsin, chymotrypsin, cysteine, glutathione or sodium sulphite. There was an increase in enzyme activity of about 50% in the soluble extracts obtained with trypsin, chymotrypsin and sodium sulphite, following the

addition of cysteine. The total β-amylase released (after cysteine activation of the extracts) was the same with all extractants. After treating the glutenin–starch with *p*-hydroxymercuribenzoate, and removing excess of reagent with bovine serum albumin, extracts with chymotrypsin had no β-amylase activity until cysteine was added.

No proteolytic activity was observed with glutenin–starch in the presence of cysteine. Moreover, cysteine released β-amylase with no measurable appearance of polypeptides in solution. By contrast there was a liberation of biuret-reacting material concomitant with the release of β-amylase when proteolytic enzymes were employed.

Our findings suggest that the latent β-amylase of wheat endosperm is bound to glutenin only by disulphide linkages. The β-amylase may be released by splitting the disulphide attachment. Sodium sulphite (as well as thiols) will break such bonds (Cecil & Wake, 1962). Or it may be released by splitting peptide bonds in the glutenin. In the latter circumstance the β-amylase would be released still attached by disulphide linkage to a fragment of glutenin. Further, it appears that only about 30 % of the sulphydryl groups associated with the active centre of the enzyme are involved in the disulphide attachment to glutenin, despite the inactivity of the bound β-amylase.

Excised wheat embryos have been allowed to germinate on moist glutenin–starch. Soluble active β-amylase appeared. This occurred only to a slight extent in control experiments where embryos were grown on glutenin–starch in which bound β-amylase had been heat-inactivated. Evidently developing wheat embryos secrete a substance that releases glutenin-bound β-amylase.

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Nature of the Configurational Changes of Serum Albumin in Acid

By G. Weber. (*Department of Biochemistry, University of Sheffield*)

Serum albumin is known to change reversibly its hydrodynamic and optical properties in the pH range 4–2. Rotational and translational diffusion studies and low-angle X-ray scattering (Luzzati, Witz & Nicolaeiff, 1961) are all in accord with a model of compact units linked by a flexible segment and capable of independent rotations. This view has now been substantiated by a study of the action of pepsin upon the rotational relaxation time ρ_h and sedimentation constant S_{20} of conjugates of bovine serum albumin with dimethylaminonaphthalene sulphochloride (NSA). At pH 7, NSA concentration $c = 0{\cdot}01$ %, ρ_h decreases from 107 to 36 μmsec. following treatment with $\frac{1}{300}$th weight of pepsin for 10 min. at 25°, pH 2–3, and subsequent neutralization. During the digestion ρh changes at first rapidly then much more slowly. In the rapid phase it reaches a value of 37–42 μmsec. independent of pepsin/albumin ratio ($\frac{1}{150}$ to $\frac{1}{700}$) and pH 1·9–3·6). Under conditions of maximum turnover 35–45 moles of albumin are converted per mole of pepsin per minute. The reaction velocity, defined as the initial rate of change in ρ_h with time, is constant over the pH range 1·9–3·5, falls to 0·3 of this value at pH 3·75 and 10^{-3} of it at pH 4·05. Thus the acid configuration change of albumin is necessary for the action of the enzyme. After pepsin action is described the rotational relaxation time becomes pH-independent but concentration dependent. At pH 7·5, $\rho_h = 58$ μmsec. at $c = 1{\cdot}4$ % and 36 μmsec. at 0·4–0·005 %. Ultracentrifugation of synthetic boundaries at pH 7 shows a single peak with S_{20} 2·3 at 0·8 %; 2·15 at 0·4 % and 2·0 at 0·2 %. The changes in ρ_h and S show that a reversible association between the split pieces obtains in solution. Two fractions, accounting together for 75 % or more of the original absorbancy at 280 mμ, have been isolated by chromatography. Preliminary values for these fractions are: $\rho_h = 19$ μmsec. $S_{20} = 1{\cdot}5$ s for fraction I; $\rho_h = 50$ μmsec. $S_{20} = 2{\cdot}6$ s for fraction II.

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Estimation of Fibrinolytic Activity in Human Plasma by Heparin Fractionation

By J. Green and W. B. Thomson. (*Department of Pathology, Paddington General Hospital, London, W. 9 and Medical Unit, St Mary's Hospital, London, W. 2*)

The finding of rapid lytic activity in a heparin fraction prepared from human plasma (Green, 1959) has been adapted for experimental study. Plasma is diluted with two volumes of water containing 12·5 units of heparin/ml. and the pH is adjusted to 6·8; the heparin complex, which contains fibrinogen and plasminogen, is spun down, washed and redissolved in barbiturate–NaCl buffer, pH 7·5, ionic strength 0·15. It can be used for visual determination of lytic activity after clotting

by thrombin, determination of proteolytic activity by measuring the extinction at 280 mμ after precipitation, and esterolytic activity towards arginine and lysine esters.

Reproducible results require a constant ratio of anticoagulant to plasma, and also that all samples be kept below 5° until they are dissolved at room temperature for testing. With a three- or four-fold excess of EDTA as initial anticoagulant, most of our lysis times at pH 7·5 and 37° fall within the range of 10–90 min., and show a close correlation with results obtained by Fearnley, Balmforth & Fearnley's (1957) whole-blood technique.

Proteolysis has been measured by modification of Anderson's (1962) technique. Amounts (1 ml.) of the dissolved complex, equal to 1 ml. of plasma, are clotted by thrombin, (4 units), and incubated at 25° for 2 hr. The increase in tyrosine-containing peptides is measured at 280 mμ after addition of urea and trichloracetic acid.

Esterase activity has been studied by Brown's (1960) method. Amounts of solution (1 ml.), equal to 2 ml. of plasma, are incubated with 20 μM-lysine ethyl ester, and 1 ml. amounts, equal to 1 ml. of plasma, with 20 μM-tosylarginine methyl ester (TAME), for 2 hr. at 25°, pH 7·5. Fractions prepared from citrated plasma have shown 10–30% hydrolysis of lysine ethyl ester (LEE), and 30–80% hydrolysis of TAME under the above conditions. Fractions from EDTA plasma show little or no esterase activity under normal conditions, but in some experiments when the mixture was clotted with thrombin, increased hydrolysis of LEE correlated with increased lytic activity. The reason for this disparity is not yet clear.

Both proteolytic and esterase methods have proved useful in studying varying activity in individuals.

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An Adenosine Triphosphatase from Electric Organ Activated by Sodium and Potassium and Inhibited by Ouabain or Oligomycin

By I. M. Glynn. (*Physiological Laboratory, University of Cambridge*)

In 1957 Skou showed that the 'microsomal' fraction from minced crab nerves contained an ATPase activated by sodium and potassium ions, and he suggested that such an enzyme might be involved in the active transport of sodium and potassium across cell membranes. Evidence for this view has been provided by experiments on red-cell membranes in which it has been shown that (i) a part of the ATPase activity occurs only when potassium is present outside the cells and sodium is present inside (Glynn, 1962), and (ii) there are close resemblances between the ATPase and the ion-transport system especially in the way they are affected by cardiac glycosides (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961).

Active transport in red cells is relatively sluggish. R. D. Keynes (personal communication) has evidence suggesting that in the electric organ of *Electrophorus electricus* ion pumping is extremely active immediately after a discharge, and it therefore seemed worthwhile to examine the ATPase activity in this organ.

A piece of electric organ was homogenized in buffered sucrose solution and the 'microsomal' fraction separated by centrifuging. This fraction was found to possess marked ATPase activity, of which more than 90% occurred only in the presence of both sodium and potassium as well as magnesium. The activity that required sodium and potassium was completely inhibited by ouabain (3×10^{-5} g./ml.) or sodium lauryl sulphate (0·5%), and 75% inhibited by oligomycin (10^{-5} g./ml.), which also inhibited Na transport in red cells. Dinitrophenol (1 mM) was without effect.

The effect of sodium lauryl sulphate suggests that membrane structure may be important. Inhibition by oligomycin suggested a possible link with 'reductive dephosphorylation' (Chance, 1961), and the effects of dithionite (1 mM), ascorbic acid (10 mM), ascorbic acid (10 mM) plus *p*-phenylenediamine (1 mM), KBH_4 (1·3 mM) and H_2O_2 (5 mM) were therefore investigated. The reducing agents caused 20–30% inhibition; H_2O_2 caused 65% inhibition but this may have been due to oxidation of sulphydryl groups, as partial or complete inhibition could be obtained with a number of 'sulphydryl reagents'.

Comparison of the absorption spectrum of the preparation in the oxidized and in the reduced state showed no trace of cytochromes, flavoproteins, ubiquinone or pyridine nucleotides.

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Vitamin A and Membrane Systems. 1. The Action of the Vitamin on the Membranes of Cells and Intracellular Particles

By J. T. Dingle,* A. M. Glauert,† M. Daniel‡ and J. A. Lucy.* (*Strangeways Research Laboratory, Cambridge*)

Studies in this laboratory on the action of vitamin A on organ cultures, erythrocytes and lysosomal particles have indicated that excess of the vitamin may decrease the stability of lipoprotein membranes. The mechanism of this effect, which shows a high degree of molecular specificity, has been investigated further.

Observations made by phase-contrast and electron microscopy show that erythrocytes assume various bizarre forms within one minute of the addition of vitamin A. Large indentations are produced and the cells often appear cup-shaped. Smaller indentations appear in the surfaces of the larger depressions, and are then pinched off to form small vacuoles within the cells; the process resembles pinocytosis. The erythrocytes then become spherical and gradually less dense as haemoglobin is lost, until empty ghosts are formed. The vacuoles persist throughout haemolysis and remain in the ghosts.

Addition of vitamin A to fibroblasts in buffered saline at 37° causes local distensions in the cell membrane which finally disintegrates; the nuclear membrane also may be damaged, although it is sometimes still recognizable when the cell membrane has completely disappeared. The cytoplasm contains numerous large granules. The mitochondria become swollen, and their internal structure distorted.

Vitamin A ($3{\cdot}5 \times 10^{-5}$ M) also causes isolated rat-liver mitochondria to swell rapidly (J. A. Lucy, M. Luscombe & J. T. Dingle, unpublished observations). This effect is temperature-dependent and shows a high degree of molecular specificity.

These observations demonstrate that excess of vitamin A disorganizes the structures of the membranes of different types of cells and of those of a number of intracellular particles. This widespread action indicates that vitamin A interacts with structural components that are common to these membranes. Changes in membrane structures may lead to a variety of effects, including alterations in metabolism (Dingle, Lucy & Fell, 1961), haemolysis (Lucy & Dingle, 1962) and degradation of cartilage matrix (Fell, Lucy & Dingle, 1961).

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Vitamin A and Membrane Systems. 2. Membrane Stability and Protein–Vitamin A–Lipid Interactions

By J. A. Lucy* and J. T. Dingle.* (*Strangeways Research Laboratory, Cambridge*)

Erythrocytes are lysed on incubation with vitamin A *in vitro*, and the molecular requirements for haemolytic activity are similar to those for the biological activity of the vitamin (Lucy & Dingle, 1962; Dingle & Lucy, 1962). Haemolytic properties are associated with a capacity to penetrate cholesterol films or disperse protein films (Schulman & Rideal, 1937); the relationship between haemolytic activity and penetration of cholesterol has been shown to hold for vitamin A (A. D. Bangham, J. T. Dingle & J. A. Lucy, unpublished observations). Observations are now reported on the ability of vitamin A to interact with protein, which extend existing knowledge obtained from studies on the transport and storage of the vitamin (Ganguly, 1960).

Vitamin A (15 μg./ml.) has relatively little haemolytic action below 37° but the subsequent addition of crystalline albumin (0·5–1%) in the cold produces lysis within 2 min. Lysis of vitamin A-sensitized erythrocytes occurs with several proteins, including histone. Albumin that has previously been saturated with vitamin A is inactive, indicating that lysis depends on combination between the vitamin and protein. Furthermore, during haemolysis, cell-bound vitamin A is released into solution, presumably as part of a protein complex. Derivatives of vitamin A that are unable to haemolyse erythrocytes at 37° do not sensitize the cells in the cold. Other haemolysins, e.g. the neutral detergent, BRIJ 35, behave similarly to vitamin A, but saturation of albumin with detergent does not reduce its ability to lyse vitamin A-sensitized erythrocytes.

Similar observations have been made on mitochondrial swelling. The vitamin has little effect in the cold, but subsequent addition of albumin results in immediate swelling.

These phenomena apparently depend on interaction of vitamin A with components of lipoprotein membranes followed by combination with

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exogenous protein, and such reactions may be important *in vivo*. As a conjectural hypothesis, it is suggested that one of the physiological roles of vitamin A may be to stabilize membranes by acting as a cross-linking agent between lipid and protein; the projecting methyl groups of the vitamin, which are suitably positioned, may bind to those of cholesterol by van der Waals forces, while its hydroxyl group may be attached to protein by hydrogen bonding.

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Transport of Iodide by Rat Small Intestine

By J. D. Acland. (*Department of Pharmacology and Therapeutics, Sheffield University, Sheffield* 10)

The central region of the rat's small intestine has been observed to transport iodide against a concentration gradient *in vivo* from its serosal to its mucosal side (Pastan, 1957). This process has been further studied *in vitro* (Acland, 1959, 1962; Acland & Illman, 1959; Acland & Johnson, 1960). It was found to be aerobic and inhibited completely by 0·1 mM-potassium perchlorate. 4 mM-Potassium cyanide caused approximately 50% inhibition but 20 mM-potassium cyanide inhibited completely. Transport of iodide was unaffected by carbimazole, 6-methyl-2-thiouracil and 2-mercaptopyrazine, a specific inhibitor of horse-radish peroxidase.

Further experiments have been carried out with a perfusion technique (Acland, 1959) using Krebs's bicarbonate buffer (Krebs & Henseleit, 1932). The inhibitory action of sodium iodoacetate (IAA) on the transport of iodide was studied. 0·01 mM-IAA caused an inhibition of approximately 25% which commenced after 20–30 min. 0·1 mM-IAA caused an inhibition of approximately 50% which commenced after 5–10 min. 1 mM-IAA inhibited completely within 5–10 min. The action of iodoacetate was compared with that of the sodium salt of *p*-chloromercuriphenylsulphonic acid (PCMPS). 0·1 mM-PCMPS caused approximately 30% inhibition and 0·67 mM-PCMPS caused approximately 75% inhibition, commencing within 5–10 min. in each case.

Transport of iodide was inhibited completely by 3 mM-Amytal and was not restored by 20 mM-sodium succinate. However, the rat small intestine possesses a low succinoxidase activity (Stanbury, 1961). Transport of iodide was unaffected by 0·01 mM-dinitrophenol and was reduced by about 30% in the presence of 0·1 mM. 0·1 mM-Dinitrophenol did not increase the inhibitory action of IAA. Digoxin (10 μM) and ouabain (10 μM) added to the fluid on the mucosal side of the intestine did not affect the rate of iodide transport.

It can be concluded that the transport of iodide requires the presence of –SH (or –S–S–) groups. It is unlikely to be linked to processes of oxidative phosphorylation beyond the dinitrophenol-sensitive step, or to the operation of an ATPase sensitive to cardiac glycosides.

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Anion Activation of the 3α-Hydroxy Steroid-Dependent Nicotinamide–Adenine Dinucleotide-Transhydrogenating System of Rat Liver

By Regina Pietruszko, Marion B. R. Gore and D. N. Baron. (*Department of Chemical Pathology, Royal Free Hospital and School of Medicine, London, W.C.* 1)

It has been found that rat liver contains an enzyme system capable of catalysing reversible transfer of hydrogen from $NADPH_2$ to NAD in presence of 3α-hydroxysteroids (Hurlock & Talalay, 1958). We have investigated this enzyme in the coupled reaction system: $5{\cdot}5 \times 10^{-2}$M-tris buffer, pH 7·5, $1{\cdot}4 \times 10^{-3}$M-NAD, $2{\cdot}2 \times 10^{-5}$M-NADP, $7{\cdot}4 \times 10^{-2}$M-nicotinamide, $1{\cdot}2 \times 10^{-5}$M-androsterone and $5{\cdot}5 \times 10^{-3}$M-glucose 6-phosphate. Addition of excess of glucose 6-phosphate dehydrogenase (Boehringer), supplied as a suspension in 3·3M-$(NH_4)_2SO_4$, generated $NADPH_2$ instantaneously. Under these conditions, we observed that the rate of androsterone-dependent hydrogen transfer from $NADPH_2$ to NAD, catalysed by partially purified enzyme from rat liver, increased on addition of further glucose 6-phosphate dehydrogenase. When excess of dialysed glucose 6-phosphate dehydrogenase was used for $NADPH_2$ generation, transhydro-

genation proceeded at approximately 5% of the maximal rate; and this rate could be restored by addition of inactivated undialysed glucose 6-phosphate dehydrogenase, or of $(NH_4)_2SO_4$, to the system.

The rate of transhydrogenation was directly proportional to the concentration of $(NH_4)_2SO_4$ in the test system, up to 0·05M. Maximal activity occurred with a final concentration of approximately 0·1M-$(NH_4)_2SO_4$.

As Li_2SO_4, Na_2SO_4 and K_2SO_4 activated similarly to $(NH_4)_2SO_4$, and NH_4Cl and NaCl were ineffective in this system, it appeared that the anion was the activator. Other activating anions, tested as sodium salts, in order of decreasing effectiveness, were selenate, oxalate, arsenate, malonate, phosphate, borate, succinate, and tungstate. Sulphate had no significant effect on the pH optimum.

The activating effect of SO_4^{2-} on $NADPH_2$–NAD transhydrogenation occurred when NADP-specific isocitrate dehydrogenase and isocitric acid (3×10^{-3}M) were used as the $NADPH_2$-generating system, when aetiocholanolone or androstanedione replaced androsterone, and also when acetylpyridine–adenine dinucleotide replaced NAD as the hydrogen acceptor.

These results indicate that the ionic composition of the test system is critical in assessing the activity of the 3α-hydroxysteroid-dependent $NADPH_2$–NAD-transhydrogenating system of rat liver. This may have a bearing on low rates of activity previously reported (1·1 μm-moles of $NADH_2$ formed/mg./min., Hurlock & Talalay, 1958) and on failure to detect this enzyme (Stein & Kaplan, 1959). Partially purified material prepared during this work catalysed the formation of 30 μm-moles of $NADH_2$/mg./min.

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Secretion of Insulin by Rabbit Pancreas *in vitro*

By H. G. Coore and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

Insulin secretion has been studied *in vitro* with small pieces of rabbit pancreas (approx. 250 mg.) incubated for up to 120 min. in 3 ml. of oxygenated bicarbonate medium supplemented with pyruvate, fumarate and glutamate. The tissue is suitable for this purpose being thin and having a relatively high concentration of β cells. The concentration of insulin in the incubation medium has been measured by isotope dilution using Hales & Randle's (1962) modification of the Yalow and Berson immunoassay.

The rate of insulin release in a typical experiment increased from 22 μunits/ml./min. at 0·7 mg./ml. of D-glucose to 265 μunits/ml./min. at 1·5 mg./ml. The magnitude of response to glucose could be compatible with that of the gland *in vivo*. The response of a particular piece of pancreas exposed to a high concentration of glucose (e.g. 2 mg./ml.) can be attained a second time when the piece of gland has been rested for 30 min. at a low glucose concentration. This reproducibility of response at different times of incubation allows each piece to be used as its own control.

D-Galactose (3 mg./ml.), D-2-deoxyglucose (3 mg./ml.) and glucagon (5 μg./ml.) failed to stimulate insulin release. Glucose (3 mg./ml.) failed to stimulate insulin secretion when pancreas was incubated anaerobically or in the presence of 0·25 mM-2,4-dinitrophenol. Tolbutamide (0·2 mg./ml.) in the presence of glucose (0·7 mg./ml.) stimulated insulin secretion tenfold. The stimulating effect of glucose at 3 mg./ml. on insulin secretion was enhanced by pyruvate (0·5 mg./ml.) but 3 mM-phloridzin did not influence the response to glucose at 2 mg./ml. We conclude that this relatively simple *in vitro* system is suitable for a study of the factors regulating insulin secretion.

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Effect *in vitro* of Fatty Acids on Glucose and Pyruvate Metabolism in the Rat Hemidiaphragm

By P. B. Garland* and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

The effects of *n*-butyrate and palmitate on glucose uptake, glycogen and glycerol 1-phosphate concentrations, output of lactate and pyruvate, and pyruvate utilization have been measured in isolated hemidiaphragms from non-starved female Wistar rats.

Butyrate (1) decreased glucose uptake with maintained or increased glycogen content, (2) increased proportion of glucose appearing as lactate, (3) decreased pyruvate uptake with an increased proportion converted into lactate, (4) increased ratio of lactate/pyruvate in medium and (5) increased glycerol 1-phosphate concentration. In addition, butyrate decreased the conversion of [1-^{14}C]glucose

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and [2-^{14}C]pyruvate into $^{14}CO_2$. Palmitate (albumin-bound) caused similar but smaller changes.

Effects (1)–(3) are similar, (4) and (5) opposite, to the behaviour *in vitro* of diaphragm muscle from alloxan-diabetic and 48 hr.-starved rats in the absence of added fatty acids.

On the basis of effects (4) and (5), their reversal in tissue from diabetic and starved rats, and of further experiments measuring the glycerol 1-phosphate/dihydroxyacetone phosphate ratio, it is suggested that regulation of the muscle intracellular glycerol 1-phosphate concentration in response to fatty acids, starvation and diabetes is dependent not only on the glycolytic supply of dihydroxyacetone phosphate, but also the cytoplasmic $NAD/NADH_2$ ratio.

Immunoassay of Insulin by Isotope Dilution

By C. N. Hales* and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

Yalow & Berson (1960) first described an immunoassay for insulin based upon the fact that unlabelled insulin can diminish the binding of [^{131}I]-insulin by insulin antibodies. The present modification, based essentially on isotope dilution, has the advantages of being simpler and quicker, and of using insulin of relatively low specific activity which is easier to handle and less subject to radiation damage. Bovine insulin (50 μg.) is iodinated with ICl (2 mc of ^{131}I) yielding [^{131}I]insulin of specific activity 5–10 mc/mg., chromatographically and electrophoretically indistinguishable from unlabelled insulin. For assay bovine [^{131}I]-insulin (approx. 200–400 $\mu\mu$g.) is mixed with a fixed amount of guinea-pig anti-human insulin serum (amount determined beforehand to ensure labelled insulin in excess) and standard or unknown insulin solutions. After 4 hr. incubation at 4°, the insulin–antibody complex is precipitated with rabbit anti-guinea-pig γ-globulin serum for 16 hr. and the precipitate collected by microfiltration and assayed for radioactivity. With anti-insulin serum in excess all the radioactivity is recovered in the precipitate. The radioactivity of the precipitate decreases with increasing concentrations of added unlabelled human insulin. It is possible to detect 0·6 μunits of human insulin and to assay 100 samples in duplicate each day.

The method has been applied to the measurement of insulin in human and rat plasma. The mean plasma insulin level obtained in 5 fasting normal individuals of 17 μunits/ml. agrees well with that of 21 μunits/ml. reported by Yalow & Berson. There was a ninefold rise following 100 g. of glucose orally. Dilution of plasma and recovery of insulin added to plasma gives satisfactory results. The insulin levels in plasma are reproducible after storage of samples in the deep freeze.

No insulin was detectable in the plasma of alloxan-diabetic rats 48 hr. after injection. The mean plasma insulin of 48 hr. starved, 5 hr. starved and fed rats were 20, 30 and 100 μunits/ml. respectively (expressed as human insulin).

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The Effect of Fatty Acids, Ketone Bodies, Starvation and Diabetes on Pyruvate Metabolism in Rat Heart

By E. A. Newsholme and P. J. Randle. (*Department of Biochemistry, University of Cambridge*)

The uptake of pyruvate by hearts from fed rats perfused for 15 min. with bicarbonate medium containing 4 mM-sodium pyruvate was 167 μmoles/g./hr. This was significantly reduced by 5·5 mM-β-hydroxybutyrate (−31 %), by 5·5 mM-acetoacetate (−51 %), by 2·8 mM-*n*-octanoate (−54 %) and by 0·75 mM-palmitate on 2 % bovine serum albumin (−28 %). Pyruvate uptake by hearts from alloxan-diabetic or starved rats (48 hr.) was depressed 38 and 45 % respectively. Lactate output was not changed significantly.

When hearts were perfused with glucose (1 mg./ml.) and insulin (0·1 unit/ml.) glucose uptake (control range 66–75 μmoles/g./hr.) was reduced approx. 50 % by alloxan-diabetes, starvation, 2·8 mM-*n*-octanoate and 0·75 mM-palmitate by inhibition of hexokinase and phosphofructokinase reactions. In spite of the reduction in glucose uptake the combined output of lactate and pyruvate (approx. 70 μmoles/g./hr.) was not diminished by diabetes or starvation and was increased by octanoate (45 %) and by palmitate (27 %). The ratio of lactate to pyruvate in the perfusion medium (9·6) was reduced by diabetes (47 %) and starvation (61 %) but increased by octanoate (200 %) and by palmitate (90 % from 4·2).

We conclude that the uptake and oxidation of pyruvate by rat heart and the oxidation of pyruvate formed from glucose is depressed by diabetes, starvation, fatty acids and ketone bodies, and that the lactate to pyruvate ratio is increased by fatty acids and depressed by diabetes and starvation. We suggest that enhanced respiration of fatty acids

is responsible for depressed pyruvate uptake and oxidation in diabetes and starvation and that some other factor leading to an alteration in the ratio of cytoplasmic NAD to $NADH_2$ is responsible for alterations in the ratio of lactate to pyruvate in these conditions.

The Effect of Adrenaline on Phosphorylase Activity, Glycogen Content, and Isotonic Tension of Intestinal Smooth Muscle (Taenia Coli) of the Guinea-Pig

By A. R. Timms, E. Bueding, J. T. Hawkins and J. Fisher. (*School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md., U.S.A.*)

Adrenaline ($5{\cdot}4 \times 10^{-7}$ M) causes a relaxation of isolated intestinal smooth muscle (taenia coli) of the guinea-pig suspended in a Krebs–bicarbonate solution (Bülbring, 1954). A simultaneous activation of phosphorylase was demonstrated by Axelsson, Bueding & Bülbring (1961). This was confirmed in the present study; in addition, a fall in the mean level of glycogen from 1·46 to 1·01 mg./g. (wet wt.) was observed 2 min. after exposure to $5{\cdot}4 \times 10^{-7}$ M-adrenaline.

After pre-exposure of the muscle to dichloroisopropylnoradrenaline ($1{\cdot}95 \times 10^{-6}$ M) for 5 min., and readjustment of the tension which had fallen owing to this agent, adrenaline ($5{\cdot}4 \times 10^{-7}$ M) still caused relaxation, but no increase in phosphorylase A occurred. Furthermore, in confirmation of Bülbring's observations (E. Bülbring, personal communication), it was found that considerably lower concentrations of adrenaline ($2{\cdot}7 \times 10^{-8}$ M) alone cause relaxation of smooth muscle (average decrease of tension from 1·0 g. to 0·35 g.); however, neither an increase in phosphorylase A activity nor reduced glycogen levels were observed.

It is concluded that the relaxant effect of low concentrations of adrenaline on guinea-pig taenia coli muscle can occur without conversion of phosphorylase B into A, which in turn could supply energy for the stabilization of the membrane (Axelsson *et al.* 1961). Since the decrease in tension produced by low concentrations of adrenaline is *not* associated with increased fructose 1,6-diphosphate concentrations nor with decreased fructose 6-phosphate levels of taenia coli, the hypothesis that activation of phosphofructokinase occurs under these conditions receives no support. On the other hand, the possibility that some other metabolic process is stimulated either directly or indirectly by adrenaline or by cyclic 3′,5′-AMP cannot be excluded, particularly because conditions known to increase metabolic activity have the same effect as adrenaline on the mechanical and electrophysiological state of taenia coli (Axelsson & Bülbring, 1961).

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The Action of Seminal Enzymes on Coenzyme A

By M. Libonati and E. Leone. (*Istituto di Biochimica Applicata, Università di Sassari, Italy*)

During a recent study of CoA pyrophosphatase in bull-seminal plasma (Libonati & Leone, 1962), we noted the presence in that fluid of a CoA peptidase. Whereas CoA pyrophosphatase resembles, and is probably identical with, the nucleotide pyrophosphatase of Kornberg & Pricer (1950), CoA peptidase has not so far been investigated in detail, except for the original description of the pigeon-liver enzyme by Novelli, Schmetz & Kaplan (1954).

The inactivation of CoA was followed by three independent methods (sulphanilamide acetylation: Kaplan & Lipmann, 1948; oxoglutarate oxidation: Von Korff, 1953; sorbyl-CoA ligase: Wakil & Hübscher, 1960). The action of the seminal peptidase was accompanied by the appearance of a positive ninhydrin reaction owing to the splitting of the β-mercaptoethylamine (MEA) fragment. An attempt was made partially to purify the peptidase and separate it from the pyrophosphatase. This was achieved by ammonium sulphate fractionation of an extract from bull-seminal plasma obtained after treatment with protamine sulphate. The fraction obtainable between 40 and 70% saturation with ammonium sulphate showed both CoA-pyrophosphatase and -peptidase activity. However, the fraction precipitated between 70 and 100% saturation possessed only peptidase activity.

The seminal peptidase responsible for splitting off the MEA fragment had an optimum pH value at 8·15. This fact has been established by paper-chromatography analysis of incubation mixtures; MEA has been identified by its R_F value which was identical with that of a sample of pure material (California Biochemical Corporation), and by the positive nitroprusside and ninhydrin reactions.

Exhaustive dialysis against 0·005M-tris buffer, pH 7·7, in the absence or presence of 10^{-3}M-EDTA, did not impair the peptidase activity. These experiments are now being extended to the seminal plasma of other animals.

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Dehydroxylation of [^{14}C]Protocatechuic Acid in the Rat

By J. C. Dacre* and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

3,4-Dihydroxyphenylacetic acid (homoprotocatechuic acid) is dehydroxylated in the rat and rabbit to *m*- and *p*-hydroxyphenylacetic acid (Booth, Murray, DeEds & Jones, 1955; Scheline, Williams & Wit, 1960). On feeding 3,4-dihydroxybenzoic acid (protocatechuic acid) to rats and rabbits, DeEds, Booth & Jones (1957) found that it was methylated to vanillic acid, but was not dehydroxylated. With [*carboxy*-^{14}C]protocatechuic acid (prepared according to Neish, 1959) we have found that this acid is dehydroxylated to a small extent in the rat.

On feeding the labelled acid (100 mg./kg.) to rats, it was found that 70 % (range 62–78 % in 6 rats) of the ^{14}C was excreted in the urine in 7 days. Chromatography of the urine showed that it contained radioactive protocatechuic, vanillic (but not isovanillic acid), *m*- and *p*-hydroxybenzoic, and *m*-methoxybenzoic acids. Preliminary quantitative estimates of the amounts of these acids excreted have been made by isotope dilution, with the following results: Protocatechuic acid, 36 %, vanillic acid, 20 %, *m*-hydroxybenzoic acid, 2 %, *p*-hydroxybenzoic acid, 1 %, and *m*-methoxybenzoic acid, 2 % of the dose. Conjugates of these acids have been allowed for in these figures. Dehydroxylation is thus a minor metabolic route (about 5 %) of protocatechuic acid in the rat, and is less than that found for homoprotocatechuic acid [8 % in rats and 15 % in rabbits; Scheline *et al.* (1960) and unpublished results].

An interesting new finding is the presence of labelled *m*-methoxybenzoic acid which could arise either by dehydroxylation of vanillic acid or methylation of *m*-hydroxybenzoic acid. In rats given neomycin (to destroy gut bacteria) for 3 days prior to administration of [^{14}C]protocatechuic acid, it was found that the ^{14}C excreted in 7 days was 86 % (range, 84–89 % in 3 rats). Chromatography of the urine showed the presence of protocatechuic, vanillic and *m*- and *p*-hydroxybenzoic acids, but no *m*-methoxybenzoic acid.

The bile from rats injected intraperitoneally with [^{14}C]protocatechuic acid (100 mg./kg.; 1·4 μC) was collected for 24 hr. by means of a bile duct cannula. The bile contained 13 % (range 11–14 % in 3 rats) of the administered ^{14}C, present mainly as a non-reducing glucuronide of vanillic acid (70 % of biliary ^{14}C).

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Inhibition of Mitochondrial Oxidation Systems by the Carcinogen, *N*-2-Fluorenyldiacetamide

By A. R. Crofts and J. B. Chappell. (*Department of Biochemistry, University of Cambridge*)

Kielley (1956, 1957) showed that 150 μM-*N*-2-fluorenyldiacetamide (FdiAA) inhibited the oxidation of glutamate by rat-liver mitochondria to a greater extent (87 %) than that of other substrates with NAD-linked dehydrogenases (about 30 %) or succinate (2 %). Added NAD relieved this inhibition. These results have been confirmed by Emmelot & Bos (1957) who suggested that the inhibitory effect was due to action on mitochondrial structure resulting in release of bound NAD.

These findings have been re-investigated using the oxygen electrode and under conditions designed to maintain mitochondrial integrity. In parallel experiments swelling was measured by following apparent extinction changes at 700 mμ. At low phosphate concentrations (5 mM) 150 μM-FdiAA produced a 40 % inhibition of the oxidation of those substrates with NAD-linked dehydrogenases, including glutamate, and a somewhat

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smaller inhibition of succinate oxidation. Maximum inhibition was observed at 250 μM-FdiAA, but, remarkably, at even higher concentrations inhibition was considerably less. These inhibitory effects have been observed when respiration was stimulated by ADP, 2,4-dinitrophenol or arsenate in the presence of ADP. With Keilin–Hartree preparations, the oxidation of reduced NAD was inhibited by 60% and that of succinate by 30% on addition of 250 μM-FdiAA. These values are similar to those observed with intact mitochondria. In no case did added NAD have an effect on the inhibition produced by FdiAA.

At higher phosphate concentrations (15 mM) the addition of 250 μM-FdiAA caused marked acceleration of mitochondrial swelling which was extensive and complete in 4 min. at 30°. At 5 mM-phosphate no marked swelling occurred. The primary effect of FdiAA appears to be on the respiratory chain, at the level of the carriers. Also, in the presence of higher concentrations of phosphate, swelling occurs resulting in further inactivation of the respiratory chain due to loss of NAD. It is suggested that a selective inhibitory effect on glutamate oxidation was observed by previous workers because the metabolism of this substrate by mitochondria is known to involve several enzymic steps (see Borst & Slater, 1960). The markedly decreased inhibition of oxidation observed with concentrations of FdiAA in excess of 250 μM is not readily explainable but may indicate the existence of an alternative electron-transport system which by-passes the site of action of this carcinogen.

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Mechanism of the Inhibition of Respiration by Glyoxylate in Liver and Kidney Particles

By A. Ruffo, Anna Adinolfi, G. Budillon and G. Capobianco. (*Laboratory of Biochemistry, Institute of Tumours, Pascale Foundation, Naples, Italy*)

Glyoxylate and oxaloacetate were found to react together non-enzymically yielding oxalomalate, which was isolated and identified as a competitive inhibitor of aconitase (Ruffo, Romano, Testa, Adinolfi & Pelizza, 1959, 1962). In order to investigate the physiological role of this reaction in animal tissues, we determined the O_2 uptake, the P/O ratio, and the eventual formation of citrate, in intact or damaged rat-liver mitochondria, and in rabbit-kidney-cyclophorase preparations, incubated with L-glutamate, oxoglutarate, succinate, pyruvate or citrate, in the presence and in the absence, either of glyoxylate (with or without oxaloacetate), or of oxalomalate. For this purpose, intact mitochondria were prepared by the usual technique in 0·25 M-sucrose, and incubated at pH 7·4 in saline isotonic media at 30°, for 25–30 min. Damaged mitochondria, prepared in 0·25 M-sucrose, and acid EDTA, were incubated for 1 hr. at 37°, in an hypotonic acidic medium. Cyclophorase was prepared and incubated according to Green, Loomis & Auerbach (1948). Phosphorylations were uncoupled by adding 3×10^{-5} M-dinitrophenol (final concentration) to intact mitochondria, or 8×10^{-5} M to cyclophorase preparations. The O_2 uptake was determined with the conventional Warburg technique, phosphates and citrate according to Fiske & Subbarow (1925) and Ettinger, Goldbaum & Smith (1952) respectively.

The results showed that small amounts of glyoxylate plus oxaloacetate always inhibited the O_2 uptake of L-glutamate, oxoglutarate or pyruvate in intact mitochondria, without a parallel decrease of the P/O ratio. The uncoupling phosphorylative reaction, either in mitochondria or in cyclophorase preparations, did not modify the inhibition produced by glyoxylate plus oxaloacetate. Whereas the addition of glyoxylate to pyruvate or citrate incubated with intact mitochondria, besides inhibition of O_2 uptake, produced an accumulation of citrate, this substance did not accumulate when the substrates were oxoglutarate or succinate. Oxalomalate inhibited the oxidation of all the substrates tested, but the inhibition, compared with that of glyoxylate, was greater with citrate and pyruvate than with oxoglutarate and succinate. Finally, it was found that the inhibitory effect of glyoxylate on the oxidation of succinate or oxoglutarate, but not on that of citrate, was abolished by the addition of equimolar concentrations of pyruvate. All these results suggest that glyoxylate in animal tissues may control the rate of the citric acid cycle, either by inhibiting aconitase by the mechanism previously suggested (Ruffo *et al.* 1959, 1962), or by interfering directly on some other step of the cycle.

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Inhibitory Effect of Glyoxylate on the Acetyl Transfer in Pigeon Liver

By A. Ruffo, Anna Adinolfi and Marta Romano. (*Department of Biological Chemistry, University of Perugia, Italy*)

Previous results showed that glyoxylate, besides inhibiting aconitase by condensation with oxaloacetate, might also interfere directly with some other step of the citric acid cycle (Ruffo, Adinolfi, Budillon & Capobianco, 1962). Because of the molecular similarity between glyoxylate and acetate, we supposed that the inhibition may occur at the level of the enzyme system related to acetyl transfer. Therefore, partially purified acetyl transferases, prepared according to Stern & Ochoa (1951) by fractionating an aged extract of acetone powder from pigeon liver with ammonium sulphate between 40 and 70% saturation, were incubated with ATP, CoA, acetate, and oxaloacetate or sulphanilamide, in the presence and in the absence of glyoxylate. At the end of the incubation, the formation of citrate, or the acetylation of sulphanilamide, was determined. Moreover, in order to study which stage of the acetyl transfer is affected by the inhibition, crystalline phosphotransacetylase, purified from *Clostridium kluyveri* according to Bergmeyer, Klotsch & Lang (1961) (Boehringer und Soehne, Tutzing, Germany) was incubated with acetyl phosphate and CoA, in the presence and in the absence of glyoxylate. At the end of the incubation, acetohydroxamic acid was determined with the method of Lipmann & Tuttle (1945).

The results show that glyoxylate, added in equimolar concentration to either acetate or oxaloacetate and incubated with the enzyme preparation from pigeon liver, produced an inhibition of citrate synthesis varying between 80 and 100%. Similar values of inhibition of the acetylation of sulphanilamide were observed when glyoxylate and equimolar concentrations of acetate were incubated with sulphanilamide. Moreover, the addition of glyoxylate to an incubation mixture containing phosphotransacetylase, CoA and acetyl phosphate also produced an inhibition of about 50% of the formation of acetyl-CoA. Since the highly purified preparation used was expected to be free of malate synthetase, the last result seems to indicate that glyoxylate may inhibit the transfer of active acetate to CoA. Further experiments to test this possibility are in progress. The results obtained show that glyoxylate inhibits the enzymic reactions which catalysed the acetyl transfer to either oxaloacetate or sulphanilamide in pigeon liver.

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Electrophoretic Separation of Lactate Dehydrogenase Isoenzymes on Cellulose Acetate

By H. Barnett (introduced by N. F. Maclagan). (*Department of Pathology, Westminster Hospital, Queen Mary's Roehampton Hospital, London, S.W.*15)

The technique of electrophoretic separation of lactate dehydrogenase isoenzymes using starch block, starch gel and agar as supporting media is by now well established. This technique has been adapted for use with cellulose acetate.

Samples are run in 0·075M-barbitone buffer, pH 8·6, at 1 mA/2·5 cm. strip. The sample is applied in the centre of the strip and run for 1 hr. and 20 min. approximately. A suitable volume is 4 μl. for a 2·5 cm. strip. A small moist chamber is prepared and a clean glass slide put at the bottom. Towards the end of the run the following reagent mixture is prepared: 1M-lactate (1 vol.), 1% NAD (1 vol.), 0·1% NBT or MTT (3 vol.) and 0·1% *N*-methylphenazonium-methosulphate (0·3 vol.). This mixture is applied as a streak to the clean glass plate. A fresh strip of cellulose acetate, of the same size as the one on which electrophoresis is performed, is allowed to soak up the mixture. (Approximately 0·3 ml. of reagent mixture is suitable for a strip of 6 cm. × 2·5 cm.). At the end of the run the electrophoresis strip is superimposed on the blank strip into which the mixture has soaked. The moist chamber is closed and incubated for up to 1 hr. Usually the reaction is very rapid and takes place within minutes. The strips are then taken out and fixed in formalin or alcohol–acetic acid.

By this method up to five clearly defined well-separated purple bands can be demonstrated. The bands are present on both strips and are usually stronger on the bottom one. The relative intensity of colour of the bands varies with the tissue homogenate examined, and is in agreement with the findings on starch and agar. Sera give equally satisfactory results.

A sixth band, of albumin mobility, is commonly observed. It is pink in colour and not NAD-dependent. It is thought to be due to direct reduction of tetrazolium salts by proteins.

Even when lactate is omitted from the mixture faint purple bands still develop. These are invariably present in the same positions as those of the

isoenzyme fractions, and correspond to them in number. The significance of this 'nothing dehydrogenase' reaction is being investigated.

If 5 cm. wide strips are used, 15 μl. of samples can be applied. The fractions can then be cut out and eluted in small quantities of solvent. These eluates have been found suitable for quantitative estimation by spectrophotometry. Differential stability of fractions and pH optima are being investigated.

Differentiation of Tissue Esterases by Starch-Gel Electrophoresis and Column Chromatography

By Ruth J. A. Kirkland (introduced by E. C. Webb). (*Department of Biochemistry, University of Cambridge*)

Hunter & Markert (1957) were the first to apply histochemical techniques to the localization of esteratic activity of proteins separated by starch-gel electrophoresis. The most widely used method involves the coupling of azo dyes with naphthol produced from the hydrolysis of naphthyl acetate. Using these techniques Paul & Fottrell (1961) examined the esterases present in the tissues of several animals and reported considerable species differences in the electrophoretic pattern obtained from a given tissue. In some species, namely mouse, rat and guinea pig, there were considerable differences between different tissues. Of the tissues examined, liver and kidney presented the most complex pattern.

In the present investigation the esterases present in liver and plasma of several species were separated by electrophoresis on starch gel using the discontinuous buffer system of Poulik (1957). Considerable species differences in the number and mobility of esterases were confirmed. Esterases are noted for the overlapping substrate specificity displayed, nevertheless each esterase does possess a characteristic substrate-specificity pattern. With the aim of identifying the esterases separated by starch-gel electrophoresis the substrate specificity was examined by detecting the activity on the gel with several substrates: α- and β-naphthyl acetates, indoxyl acetate, phenyl acetate, phenyl butyrate and acetylthiocholine. In some cases the enzymes were eluted from the gel and their identity investigated.

The component esterases in extracts of dog-, horse- and ox-liver were purified by chromatography on diethylaminoethylcellulose using an increasing salt gradient for elution. In addition to substrate specificity and inhibitor effects some kinetic properties of the partially purified esterases were studied and their identity further established. By comparison of the starch-gel electrophoretic patterns of these purified esterases with those of the tissue extracts, the identity assigned to esterases present in the tissue extracts was confirmed.

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Studies on the Biosynthesis of Carnitine

By G. Lindstedt and S. Lindstedt (introduced by Committee). (*Department of Chemistry I, Karolinska Institutet, Stockholm* 60, *Sweden*)

Methionine has been shown to be a donor of the methyl groups of carnitine (Bremer, 1961; Wolf & Berger, 1961). Various ^{14}C-labelled hypothetical precursors of the carbon chain have now been administered intraperitoneally to rats and mice. Urine and trichloroacetic acid extracts of carcass have been analysed by column chromatography with Dowex 50 and Duolite C3. When γ-[*carboxy*-^{14}C]butyrobetaine is injected, 40% of the activity appears in the urine, half of which is carnitine and the rest unchanged γ-butyrobetaine. The remaining radioactivity is found almost quantitatively in carnitine (Lindstedt & Lindstedt, 1961). From γ-*NN*-dimethylamino[*carboxy*-^{14}C]butyric acid only 0·1% of the dose is found in carnitine, while γ-amino[*carboxy*-^{14}C]butyric acid gives no radioactive carnitine (cf. Bremer, 1962). No incorporation of ^{14}C into carnitine is obtained from γ-amino-β-hydroxy[*carboxy*-^{14}C]butyric acid, [2-^{14}C]ornithine or [*amido*-^{14}C]homocarnosine. The hydroxylation of γ-butyrobetaine has been studied in homogenates and acetone-powder extracts from rat liver. The reaction requires molecular oxygen and reducing agents as cofactors (Lindstedt & Lindstedt, 1962). The enzymic activity has been purified about 50-fold.

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Distribution of Fatty Acids in the Serum Phospholipids of Pigs Reared on Differing Levels of Linoleic Acid

By W. M. F. Leat. (*School of Agriculture, University of Cambridge*)

Mead & Slaton (1956) have shown that in a deficiency of essential fatty acids an eicosatrienoic acid accumulates in tissue lipids. In an experiment where six pigs had been reared on diets containing between 0·07 and 3·5% of linoleate (expressed as a percentage of total dietary calories) it had been shown that in serum lipids this trienoic acid was confined mainly to the phospholipid fraction (Leat, 1962). By means of silicic acid chromatography and differential hydrolysis the serum phospholipids have been separated into four fractions: lecithin, lysolecithin, sphingomyelin and kephalins. The fatty acid composition of each fraction has been determined by gas–liquid chromatography.

Increasing the dietary linoleate from 0·07 to 3·52% of the calories resulted in the following changes in fatty acid pattern: (i) The eicosatrienoic acid fell from 8·9 to 1·9% in lecithin, from 3·5 to 0·4% in lysolecithin and from 7·3 to 1·7% in cephalins. The level in sphingomyelin remained constant at about 1·5%. (ii) Arachidonic acid increased from 3·4 to 9·1% in lecithin, from 1·1 to 1·6% in lysolecithin and from 5·0 to 17·6% in kephalins. The level in sphingomyelin remained constant at about 1·0%. (iii) Linoleic acid rose from 6·8 to 13·6% in lecithin, but remained fairly constant in the other fractions, lysolecithin containing about 4·0%, kephalins 5·0% and sphingomyelin 1·0%.

Each phospholipid fraction had a characteristic fatty acid pattern. In lecithin about 40% of the fatty acids were saturated and 12% polyunsaturated, whereas in lysolecithin the respective values were 55% and 3%. As the unsaturated fatty acids of egg-yolk lecithin are located in the β-position (Tattrie, 1959) it would appear that serum lysolecithin is mainly of the α-configuration. The cephalins were distinguished by the high content (20%) of polyunsaturated fatty acids. 70% of the fatty acids of sphingomyelin were saturated and 2% polyunsaturated. This fraction was characterized by the presence of large amounts (45%) of long-chain fatty acids containing 22, 23 and 24 carbon atoms.

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The Effect of Autolysis on Lipids of Green Leaves

By V. H. Booth.* (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

It is well known that, when green leaves are macerated, both carotene and chlorophyll are lost by enzymic oxidation, except possibly in the iris (Booth, 1960). A new technique, in which many leaf lipids are separated on a two-dimensional paper chromatogram (Booth, 1962), makes it possible to study other effects of maceration. Two samples of about 300 mg. of each leaf under investigation were weighed. One was extracted immediately with acetone and light petroleum. The other was ground to a pulp under buffer, and left for 0·5 hr. or other period before extraction. The extracts were applied to treated filter papers. After 2·5 hr. the two chromatograms were compared by daylight and under ultraviolet irradiation.

A conspicuous effect of pulping was the diminution in size of the chlorophyll patch on the chromatogram from the pulped sample, and the emergence of a much more polar green patch. Another visible effect was the increase of a yellow pigment more polar than β-carotene, presumably an oxidation product of the latter.

All leaves contained α-tocopherol, which rapidly disappeared from the macerate in some conditions. The tocopherol-destroying agent was inactivated by boiling, was inhibited by cyanide and by antioxidants, was aerobic, and had an optimum pH of 3·2. The system was itself destroyed in macerated leaf, and is presumed to be enzymic. The activity varied considerably in different species: in *Helianthus tuberosus* the α-tocopherol was all gone in < 1 min.; in pulped iris leaf only 10% was lost in 1 hr.; other leaves showed intermediate activity. Leaves of *Hedera helix* contained γ- and δ-tocopherols, and their rates of loss were much slower than that of α-tocopherol from the same macerated sample.

Among lipids that seemed unaffected by maceration of the leaf was ubiquinone-50. Several other lipids of unknown constitution appeared regularly on the chromatograms. Among them was a spot, having a spectral absorption maximum at 258 mμ, that appeared on chromatograms near vitamin K_1. This lipid increased in amount during the autolysis of leaves.

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The Structure of the Satellite Tobacco Necrosis Virus and its Relation to the Current Ideas of the Genetic Code

By M. E. Reichmann,* M. W. Rees and R. Markham. (*Agricultural Research Council Virus Research Unit, Cambridge*)

A large amount of indirect evidence has suggested that the amino acid sequence in proteins is determined by a related sequence of bases in a specific ribonucleic acid molecule. This work has recently been discussed in detail (Crick, Barnett, Brenner & Watts-Tobin, 1961).

On the basis of incorporation experiments *in vitro* (Matthaei, Jones, Martin & Nirenberg, 1962; Speyer, Lengyel, Basilio & Ochoa, 1962), a scheme relating nucleotide triplets to all the normally occurring amino acids has been put forward. But a direct correlation between the composition of a naturally occurring protein and its corresponding nucleic acid code has not yet been obtained. While plant viruses are very attractive models for such studies, two difficulties are encountered: (1) The amount of nucleic acid in the viruses is about ten times more than would be required to code for the protein part and, consequently, the composition of the former need not be related to that of the protein in any simple way. (2) The entire sequence of nucleotides cannot as yet be determined.

The satellite tobacco necrosis virus, which is unable to multiply in plants in the absence of another virus (Kassanis & Nixon, 1961), affords an unusual opportunity for an experimental investigation of such coding systems. We have found this virus to be small, having an S_{20} of 48 s, a D_{20} of $2{\cdot}04 \times 10^{-7}$ cm.^{2}/sec. and a RNA content of 20%, implying a molecular weight of $1{\cdot}97 \times 10^6$ for the virus and one of 394 000 for the nucleic acid. The isolated nucleic acid has an S_{20} of 13·5 s indicating that it occurs as a single molecule per virus particle. From its base composition (U = 24·9%, C = 22·1%, A = 28·0%, G = 25·0%) and molecular weight, the nucleic acid would consist of a chain of 1200 nucleotides or 400 triplets which could code for a protein of a molecular weight of not more than 45 000.

The amino acid composition of the virus protein has been determined (Spackmann, Stein & Moore, 1958) using virus material grown under several different biological conditions and the findings will be discussed in the light of the requirements of the present theories of coding.

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Use of Synthetic Polynucleotides in Deciphering the Genetic Code

By P. Lengyel, J. F. Speyer, C. Basilio, A. Wahba and S. Ochoa. (*Department of Biochemistry, New York University School of Medicine, New York* 16, *N.Y., U.S.A.*)

An experimental procedure was devised for deciphering the genetic code (Lengyel, Speyer & Ochoa, 1961; Speyer, Lengyel, Basilio & Ochoa, 1962*a*, *b*; Lengyel, Speyer, Basilio & Ochoa, 1962). A system from *Escherichia coli* consisting of high-speed supernatant and ribosomes supplemented with transfer RNA incorporated amino acids into an acid-insoluble product on addition of synthetic polyribonucleotides (Nirenberg & Matthaei, 1961). The dependence of the incorporation on added transfer RNA indicates that the synthetic polyribonucleotides affect the transfer of activated amino acid residues from transfer RNA to ribosomes and act as messenger RNA in the system. Various copolymers containing 2 or 3 kinds of nucleotides were prepared with polynucleotide phosphorylase. Poly UA (5:1) promoted the incorporation of asparagine, isoleucine, leucine, lysine, phenylalanine, and tyrosine. Poly UC (5:1) promoted the incorporation of leucine, serine, phenylalanine, and proline. Poly UG (5:1) promoted the incorporation of cysteine, glycine, leucine, phenylalanine, tryptophan, and valine. Poly UAC (6:1:1) promoted the incorporation of asparagine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, threonine, and tyrosine. Poly UAG (6:1:1) promoted the incorporation of asparagine, aspartic acid, cysteine, glutamic acid, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine. Poly UCG (6:1:1) promoted the incorporation of alanine, arginine, cysteine, glycine, leucine, phenylalanine, proline, serine, tryptophan, and valine.

A method of calculation was devised for the assignment of code triplets based on the comparison of the observed incorporation of phenylalanine relative to that of a particular amino acid with a given polymer and the calculated frequency ratio of

UUU triplets to other triplets in this polymer. Using this method, triplet code letters (of as yet unknown nucleotide sequences) have been proposed for 19 amino acids. An experimentally determined code letter for glutamine is not yet available, but one has been predicted from amino acid replacement data. Two amino acids, asparagine and leucine, have more than one code letter, indicating that the code is degenerate. All of the present code letters contain uridylic acid residues. However, non-uridylic code letters could not have been detected by the method used, and the possibility that degeneracy of the code may be more extensive than apparent at the present, to include several non-uridylic code letter assignments, is not excluded. Matthaei, Jones, Martin & Nirenberg (1962) have largely confirmed these code triplet assignments. The proposed code letters are in good agreement with amino acid replacement data in nitrous acid mutants of tobacco mosaic virus and in human-haemoglobin mutants.

Treatment of synthetic polynucleotides with nitrous acid modified their coding characteristics as expected from deamination of adenine to hypoxanthine and cytosine to uracil. These studies revealed that hypoxanthine can replace guanine in amino acid coding while xanthine cannot (Basilio, Wahba, Lengyel, Speyer & Ochoa, 1962).

If the order of nucleotides within one amino acid-specific triplet containing three kinds of nucleotides is arbitrarily assigned, substitution of the nucleotide composition of the triplets into mutational amino acid-replacement data can lead to assignment of the nucleotide order within the other code triplets (Smith, 1962; Jukes, 1962). The elucidation of the absolute nucleotide order in all triplets requires therefore the experimental determination of the sequence of nucleotides in only a few triplets.

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The Incorporation of [^{32}P]Phosphate into the Ribonucleic Acid of HeLa Cells and its Relation to Ribonucleic Acid Base Composition

By T. Spencer. (*Department of Cell Biology, John Innes Institute, Bayfordbury, Hertford, Herts.*)

Attempts have been made to determine the base composition of newly formed RNA in various cells by determining the distribution of radioactivity in the RNA nucleotides after the cells had been incubated for short periods in radioactive phosphate (Astrachan & Fisher, 1961; Kitazume, Yčas & Vincent, 1962). In the present experiments this method was tried with HeLa cells. The results obtained were compared with chemical measurements of the base composition of the DNA and of the RNA isolated from the cell nuclei and from the microsomal and supernatant fractions of the cytoplasm.

The nuclei were isolated from the HeLa cells by the technique of Fisher & Harris (1962) and the cytoplasm was separated by centrifugation into a microsomal and a supernatant fraction. The RNA fractions were purified and hydrolysed in alkali (Davidson & Smellie, 1952), and the nucleotides were separated by electrophoresis. The DNA was extracted from the isolated nuclei with phenol and sodium lauryl sulphate and hydrolysed in formic acid (Vischer & Chargaff, 1948). The bases were separated by paper chromatography in the solvent system of Wyatt (1951).

All three RNA fractions had high values (relative to adenine) for cytosine and guanine, whereas the DNA had low values for cytosine and guanine.

In the experiments in which the incorporation of radioactive phosphorus was studied, the HeLa cells were grown in suspension culture with [^{32}P]orthophosphate as the sole source of phosphorus. Samples were taken after periods of 5–60 min. incubation in the radioactive medium. The RNA of the cells was isolated and hydrolysed in alkali, and the distribution of the radioactivity in the RNA nucleotides was measured.

It was found: (1) that the specific activities of the RNA nucleotides were not equal; (2) that the distribution of the total amounts of radioactivity in the nucleotides did not reflect the base composition of the DNA at any time; (3) that the distribution of radioactivity in the nucleotides changed rapidly with time.

It is therefore difficult to see how this technique can be used reliably for determining the base composition of the newly formed RNA.

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Study of the Biosynthesis of Ribonucleic Acid in Rat- and Chicken-Cell Nuclei

By P. Mandel, M. Revel, J. D. Weill, S. Busch and P. Chambon. (*Institut de Chimie Biologique, Faculté de Médecine, and Centre de Recherches Nucléaires, Strasbourg, France*)

We used Weiss's (1960) technique to measure DNA-dependent RNA-polymerase activity in rat nuclei. Nuclear extracts of normal rat liver incorporate into RNA 78 $\mu\mu$moles of [^{14}C]ATP or 210 $\mu\mu$moles of [α-^{32}P]UTP per milligram of proteins in 10 min. at 37°. Extracts of organs synthesizing few proteins, the kidney and the brain, incorporate respectively only 35 and 15 $\mu\mu$moles of UTP per milligram.

Following a 70 % hepatectomy, liver RNA-polymerase activity rises after 6 hr., reaches a 2·4-fold increase after 12 hr. and 2·2-fold after 18 hr., and returns to normal after 48 hr. Since this increase precedes by several hours the post-hepatectomy stimulation of protein synthesis, the life span of messenger RNA seems longer in mammalian cells than in bacteria. If rats are fed after an 8-day protein deprivation, the hepatic RNA-polymerase increases rapidly: it seems, therefore, that in conditions of increased protein synthesis the enzyme activity is enhanced. X-rays (750 and 1500 r.) delivered 2–12 hr. after hepatectomy do not modify the pattern of increase of RNA-polymerase activity. Therefore X-ray inhibition *in vivo* of formation of enzyme-forming systems cannot be ascribed to a disturbance of the DNA-dependent RNA-polymerase.

We also studied RNA synthesis *in vitro* in intact kidney nuclei. Ultracentrifuge analysis in sucrose gradients of RNA isolated from nuclei incubated with [^{14}C]adenine shows the existence of several labile RNA fractions sedimenting like 27 s, 22 s, 18 s, 13 s and 8 s RNA's. The administration of nicotinamide 13 hr. before death decreases by 37 % the specific activity of a highly-labelled RNA fraction. An enhanced synthesis of diphosphopyridine nucleotide thus inhibits the biosynthesis of a labile RNA in the kidney nucleus. We investigated the nature of this labile fraction by a kinetic study of [^{14}C]adenine incorporation into the various nuclear fractions: a fraction of RNA about ten times as radioactive as the bulk of nuclear RNA leaves the nucleus during the first few minutes of incubation. The specific activity of this fraction reaches its maximum before the third minute of incubation. Thirty per cent of the total nuclear RNA leaves the nucleus in 40 min. of incubation. Chromatography of this fraction on a methylated serum-albumin column shows three fractions eluted respectively before 20 s ribosomal RNA, between 20 and 30 s RNA, and with 30 s ribosomal RNA. These values are obtained only if nuclease action is prevented. It seems thus that labile RNA's of kidney nuclei are of high molecular weight.

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The Role of the Nucleolus in the Control of Protein Synthesis

By J. Jacob and J. L. Sirlin (introduced by J. Paul). (*Institute of Animal Genetics, Edinburgh* 9)

In recent years the nucleolus has been implicated in the contribution of ribonucleic acid (RNA) for the control of cell-protein syntheses (review in Sirlin, 1962). The nature of the relationship between the nucleolus and chromosomal RNA, which must ultimately transfer the genetic information from gene deoxyribonucleic acid (DNA), is being investigated. Actinomycin, which blocks DNA-primed synthesis of RNA (Hurwitz, personal communication; Goldberg & Rabinowitz, 1962; Harbers & Müller, 1962), suppresses all nucleolar RNA turnover, as shown by radioautography (Sirlin, Jacob & Kato, 1962). Since DNA is not demonstrable in the nucleolus proper (that is, apart from the organizer), it might be preliminarily concluded, pending the outcome of current work, that the intrinsic nucleolar turnover is an amplification, perhaps involving modification, of extrinsic primer chromosomal RNA, itself primed by DNA in the chromosome. Free extrinsic RNA, dependent on chromosomal RNA, was observed in the nucleus. On first principles, the assumed primer RNA is considered the messenger RNA specified by genes. The actinomycin block eventually reduces cytoplasmic RNA turnover (Perry, personal communication). Reasons are given for considering that the nucleolar (and as a whole, nuclear) control of protein syntheses in cytoplasm is mediated mainly by this informational RNA and not by protein.

One possible aspect of a nucleolar amplification or modification of messenger RNA might be for production of transfer RNA (t-RNA), which is ultimately DNA-primed (Hartmann & Coy, 1961), as apparently is the intrinsic nucleolar RNA. There is evidence for the dependence of the cell on the nucleus for the maintenance (Naora, Naora &

Brachet, 1960) and production (Georgiev, Samarina, Mantieva & Zbarsky, 1961) of t-RNA; this does not exclude a cytoplasmic source of t-RNA as well. In fact, an increased cytoplasmic activity with cell differentiation has been shown (Ficq, 1961; J. L. Sirlin, unpublished experiments). Pseudouridine C (ψUR) is presumably a precursor of t-RNA, on account of the composition of this RNA (Dunn, 1959; Osawa, 1960), the normal occurrence of free ψU nucleotide (Sluyser & Bosch, 1962), and its incorporation as such (Goldberg & Rabinowitz, 1961). The nucleolar turnover of tritiated ψUR is considerable (Sirlin, Kato & Jones, 1961). In agreement with a nuclear involvement with t-RNA, ribonuclease (which first attacks free t-RNA) prevents the incorporation of ψUR more in the nuclei than in the cytoplasm. Furthermore, ψUR-labelled nuclear t-RNA can be chased out into the cytoplasm in the presence of excess of carrier uridine and cytidine. A 50-fold excess of ψUR over simultaneously-offered labelled uridine surprisingly stimulated its incorporation, thus masking any competition for specific ψUR sites in t-RNA; this may be explained by ψUR being a rate-limiting precursor or, perhaps, by potentiation of an inducer acting on repressor t-RNA (Kurland & Maaløe, 1962).

Another possible, but less specific, t-RNA precursor is 5-methylcytosine, since it is not incorporated as such (Borek, Mandel & Fleissner, 1962). Its incorporation kinetics (Srinivasan, 1962) are different from ψUR. The implications, and the impact on this difference, of current work with other different precursors will be discussed.

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A Subcellular Amino Acid-Incorporating System from *Saccharomyces carlsbergensis*: The Effect of Preparations of Messenger Ribonucleic Acid

By L. B. Barnett,* G. Frens and V. V. Koningsberger. (*Van't Hoff Laboratorium, State University of Utrecht, Utrecht, The Netherlands*)

A subcellular system has been isolated from the yeast *Saccharomyces carlsbergensis* incorporating [^{14}C]leucine into hot-trichloroacetic acid-insoluble material (Barnett, Frens & Koningsberger, 1962). Protoplasts of *Sacch. carlsbergensis* were prepared as previously described (de Kloet, van Wermeskerken & Koningsberger, 1960). The protoplasts were lysed either osmotically by the addition of a buffer (0·05 M-tris–HCl with $MgCl_2$, 0·01 M) in the presence of digitonin or by homogenization with a Potter–Elvehjem homogenizer. Differential centrifugation yielded a fraction sedimenting between 20 000*g* and 100 000*g*, which is referred to in the following as the 'ribosomes'. Sedimentation patterns of the ribosomes obtained by these two methods showed that ribosomes obtained by homogenization sedimented as one peak with a S_{20} of 80 s (in the tris–$MgCl_2$ buffer above), whereas the ribosomes obtained by osmotic lysis yielded two peaks in the ultracentrifuge with S_{20} of 40 s and 60 s. The latter ribosomes were found to incorporate [^{14}C]leucine more actively than the former. Incubation of these ribosomes together with the pH 5 protein precipitate from the 100 000*g* supernatant resulted in an ATP-dependent system incorporating [^{14}C]leucine into the protein fraction (insoluble in hot trichloroacetic acid). Liver 'ribosomes' and pH 5 protein precipitate were completely interchangeable with the ribosomes and pH 5 protein precipitate of the yeast protoplasts for the incorporation of the radioactive amino acid.

The incorporation of [^{14}C]leucine by the subcellular system from protoplasts was maximal at about 25°, was partially decreased by the addition of a mixture of complementary amino acids and was enhanced by the addition of small amounts (0·1 mg.) of soluble 'acceptor' or 'transfer' ribonucleic acid, whereas large amounts of this RNA decreased the incorporation.

Furthermore, a 'messenger' RNA (m-RNA) preparation was made from commercial baker's yeast according to the method of Sibatani, de Kloet, Allfrey & Mirsky (1962). This m-RNA preparation was found to exhibit the normal ultraviolet spectrum of RNA; it was hydrolysed by pancreatic ribonuclease and was highly labelled when pulse labelling experiments with ^{32}P were performed.

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Under the experimental conditions, the soluble RNA appeared to become only slightly radioactive. The addition of the m-RNA preparation to the subcellular system from yeast protoplasts increased the incorporation of [^{14}C]leucine into protein from 70–100 %. This effect was not observed with m-RNA preparations that had been digested by ribonuclease.

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The composition of the ribosomal 'chase-labelled' RNA was

$$A/U/C/G = 10/8{\cdot}1/7{\cdot}3/10{\cdot}3$$

and is similar to the total root-tip RNA (Heyes, 1960):

$$A/U/C/G = 10/9{\cdot}3/8{\cdot}9/11{\cdot}7.$$

These experiments show the presence of an RNA component with a high rate of turnover and a base composition similar to DNA—properties which are characteristic of messenger RNA.

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Demonstration of the Presence of Messenger Ribonucleic Acid in Pea Seedlings

By U. E. Loening. (*Department of Botany, University of Edinburgh*)

In a fractionation procedure previously described (Loening, 1961), free ribosomes and membranous microsomal components were isolated from pea-seedling root tips. The incorporation of ^{32}P into the RNA of these fractions has now been studied using electrophoresis on cellulose acetate membrane and scanning of the strips for radioactivity and ultraviolet absorption (Loening, 1962). It was found that the structural RNA of the free ribosomes does not become labelled for at least 40 min. during incubation of the seedlings in ^{32}P at 25°. However, an RNA component associated with ribosomes isolated in the presence of 5 mM-$MgCl_2$ does become labelled within a few minutes of incubation and travels 20 % slower than the ribosomes on electrophoresis in tris buffer at pH 7·4 containing 0·1 mM-$MgCl_2$. This component could be partially 'chased' out by incubation of the seedlings in ^{31}P for up to 30 min. after a 'pulse' incubation in ^{32}P for 10 min.

The base compositions of the 'pulse-labelled' RNA (15 min. in ^{32}P) and of the 'chase-labelled' RNA (10 min. in ^{32}P and 80 min. in ^{31}P) were determined by radioactivity measurements of the nucleotides obtained by alkaline hydrolysis. The method of Cox & Arnstein (1962) was used for purifying the RNA; other methods failed to remove traces of radioactive impurities. The composition of the 'pulse-labelled' RNA was

$$A/U/C/G = 10/8{\cdot}4/6{\cdot}8/6{\cdot}6,$$

which is closely similar to the published composition of the DNA of the tissue (Heyes, 1960):

$$A/T/C/G = 10/8{\cdot}9/6{\cdot}2/6{\cdot}5.$$

The Stimulation of Protein Synthesis in a Cell-Free System by Encephalomyocarditis-Virus Ribonucleic Acid

By I. M. Kerr, E. M. Martin and T. S. Work. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

In connexion with the work which has been done on the replication of encephalomyocarditis virus (EMC) in Krebs II mouse ascites-tumour cells (Martin & Work, 1961, 1962) methods have been worked out for the isolation of a cell-free microsome–cell-sap system from these cells, which is capable of appreciable protein synthesis (Martin, Malec, Coote & Work, 1961), and it seemed of interest to test the effect of the addition of reasonably-high-titre infective EMC RNA (prepared by a hot-phenol-extraction technique from crystalline virus) to such systems obtained from infected and control cells.

The addition of virus RNA to a microsome–cell-sap system from control cells had little effect. But working with a microsome–cell-sap system from 4·25 hr. infected cells the addition of virus RNA to 50 μg./ml. resulted in a 100 % stimulation of protein synthesis in the system, thus raising it from the depressed level reached at this stage in infection to that of an optimally working control system. In contrast the addition of ascites-cell RNA or rabbit-reticulocyte ribosomal RNA had no significant effect. Various combinations of the infected and control cell fractions were then incubated in the presence and absence of added virus RNA. The greatest stimulation, threefold, was obtained with an infected microsome plus control cell-sap system.

From the results of experiments of this type it would appear that the process of infection markedly reduces with time the capacity of both the microsome and the cell-sap fractions of the infected cell to function in protein synthesis. The addition of

virus RNA appears to reverse this effect in the case of the microsome fraction but to have no effect on the cell sap.

These results are in accord with, though they very far from prove, the hypotheses that the ribosomes of infected cells are deficient in normal messenger RNA, that virus RNA or a portion of it function as a direct stimulus for the synthesis of virus protein, and that there is no requirement for virus-specific cell-sap enzymes in the synthesis of virus protein.

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Indirect Evidence for a Messenger Ribonucleic Acid Active in the Cell-Free Synthesis of Haemoglobin

By J. KRUH, J. C. DREYFUS and G. SCHAPIRA. (*Laboratoire de Recherches de Biochimie Médicale, Hôpital des Enfants-Malades, 149 Rue de Sèvres, Paris, France*)

Ribosomes from rabbit reticulocytes are able to synthesize haemoglobin when they are incubated either with pH 5 precipitate or with pH 5 supernatant from the reticulocyte soluble fraction. If RNA prepared by phenol extraction of reticulocytes or liver from rabbit is added to the system *in vitro*, the synthesis of haemoglobin is increased by 1·3 to 14 times above the control, whereas the incorporation of the radioactive precursor into the ribosomal proteins is significantly decreased. These results raise several problems: (1) Is the extract acting by its RNA or by an impurity? If RNA is previously treated with ribonuclease which is then eliminated by bentonite, the extract is inactive. (2) Is the action of RNA due to transfer RNA? When s-RNA–[^{14}C]valine is incubated with ribosomes from reticulocytes in presence of [^{12}C]valine, there is a synthesis of radioactive haemoglobin. Addition of RNA increases the synthesis by 30 %. If it had acted by its transfer RNA, there would have been a decrease of synthesis of radioactive haemoglobin by dilution, which is not the case. Furthermore, RNA is activating haemoglobin synthesis as well with pH 5 precipitate as with pH 5 supernatant although their s-RNA contents are very different. (3) Is RNA a liberating factor for haemoglobin? A cell-free system was incubated 8 min., then an excess of [^{12}C]valine was added and the incubation continued for 50 min. It was previously demonstrated (Kruh, Dreyfus, Rosa & Schapira, 1961, 1962) that in such conditions there is no more synthesis but liberation of radioactive haemoglobin from the ribosomes. The radioactivity of haemoglobin is not increased when RNA is added at the same time as [^{12}C]valine, but it is increased when RNA is added at the beginning of the incubation. RNA therefore does not act as a liberating factor.

RNA seems to act as a template for haemoglobin synthesis, decreasing the incorporation of the amino acids into the ribosomal proteins and increasing its incorporation into haemoglobin. It is suggested that the RNA from rabbit liver and reticulocytes contains messenger RNA for haemoglobin synthesis.

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The Effect of Ribosomal Ribonucleic Acid and Polyuridylic Acid on the Amino Acid Incorporation by Rabbit-Reticulocyte Ribosomes

By H. R. V. ARNSTEIN, R. A. COX and J. A. HUNT. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Following the discovery by Nirenberg & Matthaei (1961) that polyuridylic acid induces the biosynthesis of polyphenylalanine by ribosomes of *Escherichia coli*, various synthetic polynucleotides were found to stimulate the incorporation of other amino acids (Martin, Matthaei, Jones & Nirenberg, 1961; Speyer, Lengyel, Basilio & Ochoa, 1962). In a cell-free system consisting entirely of fractions from rat liver polyuridylic acid was, however, inactive (Lengyel, Speyer & Ochoa, 1961) and the question thus arose to what extent the polynucleotide code might be a special feature of the bacterial system.

We have now found that rabbit-reticulocyte ribosomes and pH 5 enzymes, isolated as described by Schweet, Lamfrom & Allen (1958), synthesize polyphenylalanine when polyuridylic acid is added. In a typical experiment, ribosomes (2 mg.), pH 5 enzymes (2 mg. of protein), glutathione (5 μmoles), $MgCl_2$ (8 μmole), KCl (40 μmole), pyruvate kinase (70 μg.), phosphoenolpyruvate (10 μmoles), ATP (0·5 μmoles), GTP (0·125 μmoles) and a mixture of 19 unlabelled amino acids (Schweet *et al.* 1958) and L-[^{14}C]phenylalanine (0·4 μc, 1·5 μg.) in 0·8 ml. were incubated at 37° for 1 hr. After cooling, carrier protein (16 mg.) was added, followed by 30 % trichloroacetic acid (0·5 vol.). The precipitated protein was isolated, purified by extraction with hot

10% trichloroacetic acid, reprecipitation from N-NaOH with trichloroacetic acid, and washing with hot ethanol and ether. Addition of polyuridylic acid (50 μg.) to the incubation mixture increased the specific radioactivity of the protein from 364 to 7710 μmc/g. In the absence of the ATP-generating mixture the radioactivity was 26 μmc/g. A similar 20- to 30-fold stimulation of phenylalanine incorporation by polyuridylic acid was obtained both with fresh and preincubated ribosomes. The incorporation of a mixture of 16 [^{14}C]-amino acids (Ala, Arg, Asp, Gly, Glu, His, Ileu, Leu, Met, Pro, Ser, Lys, Thr, Try, Tyr and Val) and unlabelled phenylalanine into protein was not increased significantly by the addition of polyuridylic acid.

The effect of polyuridylic acid was strikingly dependent on Mg^{2+} (optimum about 0·008M) and was maximum after about 15 min. incubation.

RNA isolated from rabbit-reticulocyte ribosomes (Cox & Arnstein, 1962) also stimulated the incorporation of phenylalanine. Although the effect was small (20–50%), it was similar to that of polyuridylic acid in its dependence on Mg^{2+}. The incorporation of phenylalanine induced by polyuridylic acid alone plus the incorporation due to RNA alone was, if anything, exceeded when both polyuridylic acid and either ribosomal RNA or RNA from turnip yellow mosaic virus were added. It is inferred that, under these experimental conditions, there was no competition between RNA and polyuridylic acid.

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Studies on the Mechanism of the Interaction between Soluble Ribonucleic Acid and Rat-Liver Microsomes

By H. Bloemendal and L. Bosch (introduced by Committee). (*Antoni Van Leeuwenhoek-Huis, Sarphatistraat* 108, *Amsterdam, The Netherlands*)

The metabolic interaction between soluble RNA (s-RNA) and rat-liver microsomes has been studied by following the transfer of nucleotide label from s-[^{32}P]RNA to an (unknown) microsomal RNA acceptor. This transfer reaction does not depend on polypeptide formation as may be concluded from two observations: (*a*) s-RNA, stripped of its amino acids, is still able to transfer nucleotide label to microsomal acceptor-RNA; (*b*) puromycin does not affect the GTP-dependent nucleotide transfer in concentrations which are strongly inhibitory for amino acid incorporation.

The terminal adenosine of s-RNA becomes incorporated into microsomal RNA in an internal position whereas removal of this nucleoside by periodate oxidation and cyclohexylamine treatment abolishes all transferring abilities.

Both ATP and GTP (but not UTP and CTP) stimulate the reaction but in an independent fashion. Two possible interpretations will be considered: (1) s-RNA contains two classes of polynucleotides, both able to interact with the microsomes but differing in their response to either one of the nucleoside triphosphates. (2) Only s-RNA molecules carrying the intact terminal structure are able to interact, provided GTP is present. ATP preserves or restores this structural integrity.

Following incubation of s-[^{32}P]RNA with microsomes under optimum conditions, at least 70% of the counts originally added may be recovered from the non-particulate fraction; under these conditions the soluble polynucleotides seem to remain functionally intact. Assuming that only one class of s-RNA molecules is involved in the transfer reactions mentioned above, these data may suggest that the metabolic interaction of s-RNA with the microsomes comprises a cyclic exchange of nucleotide material, possibly located in terminal positions of s-RNA.

The fact that puromycin blocks amino acid incorporation without affecting the GTP-dependent exchange of nucleotide label excludes the possibility that the GTP requirement of the latter reaction is a consequence of such a requirement for polypeptide formation.

Studies on Nucleic Acids in Tumours

By B. Tankó. (*Biochemical Institute, Medical University, Debrecen, Hungary*)

Some of the present experiments refer to isolation of RNA from livers of rabbits bearing Brown–Pearce tumour. The method of Grinnan & Mosher (1951) was modified (Zsindely, Szabolcs & Tankó, 1959) to improve yield and purity of preparations. It was observed that guanidine hydrochloride in a final concentration of 3M was required to separate

RNA from the proteins in case of normal rabbits while 2M was sufficient when using animals with tumours. Very similar results were obtained with kidney, where the molarity of the reagent had to be decreased to 1·5M to demonstrate the striking difference between controls and tumour-bearing animals. No such differences were found with spleen which is generally regarded as resistant to metastases. The results seem to indicate that the bonds between RNA and protein or possibly their structure have changed in the liver and kidney of animals bearing Brown–Pearce tumour. The base composition of the RNA preparations proved to be the same when determined in the conventional way.

In other experiments the new ascites lymphoma NK/Ly of mice described by Németh & Kellner (1960) was studied and the changes of RNA and DNA levels followed after treatment with carcinostatic agents. Three days after administration of mercaptopurine, Carcinofilin or Degranol a decrease of DNA was observed in agreement with recent findings of Fodor, Clarke & Bodansky (1961) on sarcoma 180 of mice, using mercaptopurin. RNA was at the same time increased. A new anticancer agent from Vinca Rosea caused a steady decrease of DNA from the 6th hour during 4 days, whereas the RNA was not significantly changed. A similar effect of a new drug, R 52 (synthesized by Professor L. Vargha) on solid Yoshida tumours of rats was observed.

The results suggest that the carcinostatic agents influence the nucleic acid metabolism of tumours, yet closer investigation in the conventional way of the RNA and DNA fractions revealed no difference in base composition. In case of NK/Ly the analyses resembled the data published by Kleinschmidt (1959) on ascites tumours of mice. It is felt that methods for preparing homogeneous nucleic acid fractions and investigating the nucleotide sequence would greatly help the approach to the basic problems of malignant growth.

We are indebted to Dr S. Braun, Professors B. Kellner, L. Németh and J. Baló (Budapest) who supplied us with the tumours. This work was supported by the Hungarian Academy of Sciences.

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Alternative Pathways for the Methylation of Homocysteine by *Escherichia coli*

By J. R. Guest, S. Friedman and M. A. Foster. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Investigations with enzymic extracts of several mutants of *Escherichia coli* requiring methionine for growth have established alternative pathways of the methylation of homocysteine (Guest & Woods, 1961; Foster, Tejerina & Woods, 1961, 1962). One mechanism is independent of cobalamin and requires tetrahydropteroyltriglutamate (H_4PtG_3) as coenzyme; the monoglutamate (H_4PtG) is inactive. N^5N^{10}-Methylene-H_4PtG_3 is formed with serine or formaldehyde as one-carbon donor and is converted into a relatively stable intermediate (X_3) by enzyme A (present in strain 121/176, but absent from strain 3/62). Enzyme B (present in strain 3/62, but not in 121/176) catalyses a reaction between the intermediate and homocysteine yielding methionine. The second pathway differs in that cobalamin is required and H_4PtG as well as H_4PtG_3 acts as coenzyme. Enzyme A converts either N^5N^{10}-methylene-H_4PtG or the corresponding triglutamate derivative into (presumably) analogous intermediates (X_1 and X_3) which react with homocysteine to form methionine in the presence of crude preparations of a cobamide enzyme (Foster, Jones & Woods, 1961) which can be obtained from either strain 121/176 or 3/62, but only if they have been grown in the presence of cobalamin. Enzyme B, which is not a cobamide enzyme, can only achieve this reaction with the intermediate formed from the triglutamate.

Formation of the intermediate (X_1) from N^5,N^{10}-methyleneH_4PtG by enzyme A requires FAD and $NADH_2$. Material isolated by chromatography on TEAE-cellulose reacted similarly to a synthetic product isolated after reduction of N^5N^{10}-methylene-H_4PtG with $NaBH_4$ and accepted as being N^5-methyl-H_4PtG (Donaldson & Kereztesy, 1961; Sakami & Ukstins, 1961). The analogous intermediate (X_3) which is formed enzymically and by borohydride reduction from the triglutamate is presumably N^5-methyl H_4PtG_3.

Purified enzyme B catalyses the transfer of the methyl group of N^5-methyl-H_4PtG_3 to homocysteine even in air and requires only Mg^{2+}; there is no reaction with the methyl monoglutamate derivative. The transfer of the methyl group from the monoglutamate is however more complex. Purified cobamide enzyme (Foster *et al.* 1961) is essential, but additional enzymes (present in either strain) are required as are also $NADH_2$, FAD, ATP and Mg^{2+}. There is clearly a fundamental difference in the mechanism of transfer to homocysteine of the methyl group derived from mono- and tri-glutamyl

forms of folic acid and the evidence for alternative pathways in the methylation of homocysteine by *E. coli* is thereby strengthened.

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The Photoassimilation of Acetate to Glutamate in Washed Cell Suspensions of *Rhodospirillum rubrum*

By D. S. Hoare. (*A.R.C. Unit for Microbiology, University of Sheffield*)

An analysis of the 'soluble fraction' of washed cell suspensions of *Rhodospirillum rubrum* exposed to [1-^{14}C]- and [2-^{14}C]-acetate in the light for short periods (5–240 sec.) showed that the major radioactive constituent was glutamate (Hoare, 1962). Under the experimental conditions in which the cells were 'equilibrated' in the light in the presence of acetate before addition of [^{14}C]acetate, glutamate formation from acetate was dependent on the presence of bicarbonate. The [^{14}C]glutamate samples of the soluble fraction of cells exposed for short periods to (i) [1-^{14}C]acetate in the presence of $NaHCO_3$, (ii) [2-^{14}C]acetate in the presence of $NaHCO_3$ and (iii) $Na_2{}^{14}CO_3$ in the presence of acetate have been degraded. Glutamate was decarboxylated by reaction with Chloramine-T (Cohen, 1939; Kemble & McPherson, 1954), the β-cyanopropionic acid thus obtained was converted into succinate, which was decarboxylated with a washed cell suspension of *Veillonella gazogenes* (Swim & Krampitz, 1954); the resulting propionate was degraded by the Schmidt reaction (Phares, 1951). Carbonate-C is incorporated into C-1 of glutamate, the carboxyl carbon of acetate is incorporated into C-2 and C-5 of glutamate, and the methyl carbon of acetate is incorporated into C-3 and C-4 of glutamate. This pattern of labelling has so far not been found in any other microorganism. The results are in agreement with those of Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm (1951), who partly degraded the glutamate of the cell proteins derived from cells grown in the presence of acetate and bicarbonate. The more complete degradation reported here does, however, exclude the operation of the tricarboxylic acid cycle. This is consistent with the observations on the flow of acetate carbon into the soluble components of the cell where glutamate formation precedes the formation of intermediates of the tricarboxylic acid cycle.

In accordance with Benedict (1962) it has been found that [^{14}C]acetate is incorporated into the soluble fraction of *R. rubrum* in the absence of bicarbonate provided acetate is absent during the equilibration period. Nevertheless, after 1 min. exposure the main radioactive component of the soluble fraction is glutamate. The cell-residue fraction also contained an appreciable amount of radioactivity which was associated almost entirely with material resistant to digestion with alkaline hypochlorite (Williamson & Wilkinson, 1958) and soluble in hot chloroform and presumed to be poly-β-hydroxybutyrate (cf. Stanier, Duodoroff, Kunisawa & Contopoulou, 1959).

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3-Oxoglutarate, an Intermediate in the Oxidation of Phenylacetic Acid by a *Pseudomonas*

By S. Dagley, J. M. Wood and P. J. Chapman. (*Department of Biochemistry, University of Leeds*)

Although homogentisic acid (2,5-dihydroxyphenylacetic acid) is a reaction intermediate in the oxidation of phenylalanine and phenylacetic acid by some bacteria (Chapman & Dagley, 1960, 1962), it appeared from studies by Kunita (1955) using whole cells that certain other species may convert phenylacetic acid into homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid) before ring-fission. We have isolated a *Pseudomonas* from which, after growth with phenylacetic acid, cell-free extracts were prepared that catalysed the oxidative ring-fission of homoprotocatechuic acid to give a yellow solution of a keto acid having $\lambda_{max.}$ 320 mμ in acid and 380 mμ in alkali, the changes in spectrum being freely reversible. This reaction resembles the enzymic oxidation of protocatechuic acid

to β-carboxy-α-hydroxymuconic semialdehyde (Trippett, Dagley & Stopher, 1960). It was suggested that the last-named compound is metabolized by oxidation of the aldehyde group to carboxyl, addition of water to an ethylenic bond and then an aldolase cleavage to give, as one of the products, oxaloacetate (Dagley, Evans & Ribbons, 1960). If the degradation of homoprotocatechuate followed a similar sequence the product corresponding to oxaloacetate would be 3-oxoglutarate. The following observations suggest that this is the case: (1) Cells grown with phenylacetate contained a 3-oxoglutarate decarboxylase which catalysed the anaerobic evolution of 1 mole of CO_2 per mole of substrate and was stimulated by addition of thiamine pyrophosphate plus Mg^{2+} ions. A similar enzyme was extracted from cells grown with 3-oxoglutarate as carbon source: its pH optimum was 5·7 and its product, presumably acetoacetate, was hydrolysed immediately by crude extracts. (2) Protocatechuate-grown cells contained no 3-oxoglutarate decarboxylase. (3) Homoprotocatechuate was attacked by crude extracts of phenylacetate-grown cells with an uptake of 1 mole of O_2 per mole of substrate, complete after 25 min.; evolution of 1 mole of CO_2 was slower and complete after 40 min. At 25 min. a compound was present that coupled with diazotized *p*-nitroaniline to give a derivative spectroscopically identical with that formed by 3-oxoglutarate.

In the first reaction of the suggested sequence, the benzene nucleus does not open between carbon atoms that carry hydroxyl groups. However, when this same *Pseudomonas* is grown with phenol or *p*-hydroxybenzoic acid the nuclei of catechol and protocatechuic acid, respectively, are opened by reactions of this type, since extracts convert the substrates into 3-oxoadipate and no derivatives of muconic semialdehyde are formed.

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The Bacterial Metabolism of Tartaric Acid: a New Pathway

By S. DAGLEY and P. W. TRUDGILL. (*Department of Biochemistry, University of Leeds*)

Barker (1936) suggested that *Aerobacter aerogenes* attacked tartrate by removal of water to give oxaloacetate. This was confirmed by later work using various species of *Pseudomonas* (Shilo, 1957; Shilo & Stanier, 1957), although it proved difficult to isolate the oxaloacetate because a decarboxylase was also induced in the bacteria, rapidly converting oxaloacetate to pyruvate (Krampitz & Lynen, 1956; LaRivière, 1956). By contrast, we find that *Pseudomonas* (A) (Bachrach, 1957; Dagley, Trudgill & Callely, 1961) when grown aerobically with DL-tartrate as sole carbon source yields cell-free extracts that contain very little oxaloacetate decarboxylase: no more, for example, than those from cells grown with various other carbon sources such as succinate or glycine. This indication of a different metabolic route was supported by the observations that (1) tartrate-grown cells, like those grown with glycine, oxidized glyceric acid rapidly; (2) when oxidation of D-tartrate was partially inhibited by arsenite or pyridine-3-sulphonic acid pyruvate accumulated but no other keto acid was detected. Cell-free extracts of tartrate-grown cells, although containing other active enzymes, did not attack tartrate, whereas previous workers found rapid non-oxidative decomposition of this substrate. We therefore exposed growing cultures to DL-[1,4-$^{14}C_2$]tartrate for intervals of 2, 5, 10 sec. and longer, using chromatography and radioautography to study the distribution of ^{14}C in compounds extracted from the cells with hot ethanol (Kornberg, 1958). Radioactivity appeared first in glycerate and soon afterwards in malate; subsequently the percentage radioactivity in these two compounds fell and that in lactate and alanine rose.

It is proposed that in these experiments D-tartrate lost CO_2 to give glycerate which was then metabolized by the reactions utilized by this strain when it is grown with glycine as carbon source (Dagley *et al.* 1961). Anaerobically, *Pseudomonas* (A) did not decarboxylate tartrate but, when air was admitted, CO_2 evolution greatly exceeded O_2 uptake in the first 5 min., and pyruvate and glycerate, but not oxaloacetate, were detected at this time. Changes in the amounts of various enzymes were measured when *Pseudomonas* (A) was transferred from tartrate to other carbon sources. Thus, induction of large amounts of glyoxylate carboligase and tartronic semialdehyde reductase accompanied growth with glycine or tartronic acid. Since these enzymes were not detected in cells grown with tartrate it is unlikely that tartrate is converted into glyoxate from which glycerate is then synthesized.

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The Characterization of Riboflavin Metabolite (RM1) as 9-(2′-Hydroxyethyl)-6,7-dimethylisoalloxazine

By E. C. Owen. (*The Hannah Dairy Research Institute, Ayr*)

Crossland, Owen & Proudfoot (1958*a*, *b*), while measuring riboflavin biosynthesis, encountered riboflavin metabolites in goat milk and urine. One of these, RM1, forms a single acetyl derivative. Borate does not increase its electrophoretic mobility nor does periodate react with it (Owen, Montgomery & Proudfoot, 1962). These facts imply the presence of a single hydroxyl group. RM1, like riboflavin and lumiflavin, catalyses the photolysis of methionine or EDTA (Owen *et al.* 1962). Its absorption spectrum from ultraviolet to visible is almost identical with that of lumiflavin from which several paper chromatographic systems fail to separate it. Unlike lumiflavin, RM1 passes almost completely into water from dilute chloroform solution. Owen *et al.* (1962) accordingly suggested that RM1 is 9-(2′-hydroxyethyl)-6,7-dimethylisoalloxazine which has been synthesized by Fall & Petering (1956) and by Cresswell & Wood (1960). A comparison of RM1 with Cresswell and Wood's compound (CW) follows: Crystals of both compounds from glacial acetic acid are yellow under the microscope (Owen, 1962) and pleochroic in plane-polarized light, the pleochroism being accompanied by marked changes of refractive index as the stage is rotated. I have found these same effects in lumiflavin and riboflavin. RM1 and CW are strongly birefringent and extinguish at 17° to their length. Both have their fluorescence reversibly removed by dithionite. Purified by solution in phenol and transferred again to water by adding ether, RM1 and CW have superposable spectra in chloroform acidified with glacial acetic acid, and in water both spectra change in the same way on change of pH from 1 to 13 (Owen, unpublished work; Cresswell & Wood, 1960). The R_F values (descending) of RM1 and CW (0·59–0·63) in the *n*-butanol system of Crammer (1948) or in water-saturated isoamyl alcohol (0·42–0·43) are the same, as also are those of their acetyl derivatives (butanol, 0·70–0·73; isoamyl alcohol, 0·54–0·56). The isoamyl alcohol system but not the butanol resolves either substance from lumiflavin. Both become non-fluorescent when treated with dimethyl sulphate in alkali, presumably (Kuhn & Rudy, 1936) due to methylation at N-3. In the butanol system 8-(2′-hydroxyethyl)-6,7-dimethyllumazine has an $R_F = 0{\cdot}47$ and 6,7-dimethyl-8-D-ribityllumazine has one of 0·30; in isoamyl alcohol the R_F value of both is 0·81. Unlike RM1 and CW neither of the lumazines has an absorption peak at as great a wavelength as 450 mμ but the ribityllumazine may be an intermediate in riboflavin biosynthesis (Plaut, 1961). The foregoing tests confirm the belief that RM1 is 9-(2′-hydroxyethyl)-6,7-dimethylisoalloxazine.

I am grateful to Dr H. C. S. Wood of the Royal College of Science and Technology, Glasgow, for gifts of the isoalloxazine and the two lumazines.

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The Effect of Amino Sugars and Glucose on Amino Sugar Metabolism in *Bacillus subtilis*

By C. J. Bates* and C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

D-Glucosamine 6-phosphate is a key intermediate in the biosynthesis and breakdown of amino sugars (Roseman, 1959). It is synthesized by the enzyme L-glutamine D-fructose 6-phosphate aminotransferase, EC 2.6.1.16 (Ghosh, Blumenthal, Davidson & Roseman, 1960) and is degraded by 2-amino-2-deoxy-D-glucose 6-phosphate ketol isomerase (deaminating), EC 5.3.1.10 (Roseman, 1959; Strange & Dark, 1960). Addition of *N*-acetyl-D-glucosamine to the growth medium of *Bacillus subtilis* NCTC 1379 causes repression of the first enzyme (termed synthetase) and induction of the second enzyme (termed deaminase) (Clarke & Pasternak, 1961, 1962). D-Glucosamine, *N*-formyl-D-glucosamine and *N*-propionyl-D-glucosamine have been found to initiate the same effects, though to a lesser extent. The relation between the repression of synthetase and the induction of deaminase was not constant for the amino sugars tested. This indicates that repression of synthetase and induction of deaminase are independent processes. Derivatives of D-glucosamine with larger *N*-acyl side-chains or with a different configuration in the pyranose ring were inactive.

The addition of D-glucose to *B. subtilis* growing in the presence of *N*-acetyl-D-glucosamine causes

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the induced synthesis of deaminase to be decreased (Clarke & Pasternak, 1962). At the same time the repression of synthetase was partly relieved. Several other sugars had a similar effect as D-glucose in reversing the changes in enzyme synthesis caused by *N*-acetyl-D-glucosamine. Those that were active also inhibited the incorporation of *N*-acetyl-D-[1-^{14}C]glucosamine into growing cultures of *B. subtilis*. A slower uptake of *N*-acetyl-D-glucosamine from growth media containing D-glucose may therefore account for a part of the glucose effect (cf. Magasanik, 1961).

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Repressors of Sulphate Activation in *Escherichia coli*

By R. J. Ellis* and C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

The formation of the enzymes EC 2.7.7.4 and 2.7.1.25 catalysing the synthesis of 3′-phosphoadenylyl sulphate (PAPS) from sulphate and ATP (Lipmann, 1958) in *Escherichia coli* ATCC 9723 is repressed by addition of L-cystine to the growth medium, but not by the addition of glutathione or inorganic sulphate (Pasternak, 1961, 1962). It has now been found that the sodium salts of sulphite, thiosulphate and sulphide, as well as L-methionine, also repress the PAPS-forming system when added to growth media containing sulphate. Of these compounds sulphide was found to be the best repressing agent although none was as active as L-cystine (complete repression by 100 μM-sulphide under conditions where 25 μM-L-cystine gave > 99% repression).

Two mutants of *E. coli* ATCC 9723 which were unable to utilize sulphate or sulphite for growth were grown on media containing glutathione, and extracts were tested for their ability to synthesize PAPS. Like the extracts from the parent strain, the mutant extracts contained the enzymes necessary for PAPS synthesis. Addition of sodium sulphite to the growth medium repressed enzyme formation by the parent strain but was without effect on PAPS synthesis by the mutants. It therefore appears probable that the compounds which repress in the parent strain are converted into a common metabolite such as cysteine which is the actual repressing agent. A similar situation has been cited for intermediates of other biosynthetic sequences (Davis, 1961).

These results suggest that the first stage in the synthesis of cysteine, namely the reduction of sulphate to sulphite, as well as the hydrolysis of glutathione, are slow rate-limiting reactions whereas the reduction of sulphite to sulphide and the conversion of sulphide and methionine into cysteine are processes rapid enough to give an intracellular concentration of cysteine sufficiently high to cause repression.

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Effect of Environmental Conditions on the Endogenous Metabolism of *Escherichia coli*

By E. A. Dawes and D. W. Ribbons. (*Department of Biochemistry, University of Glasgow*)

The endogenous Q_{O_2} of washed suspensions of *Escherichia coli*, grown in glucose–ammonium salts medium, varies with the phase of growth from which they are harvested. This can be correlated with the carbohydrate content of the cells, e.g. high endogenous Q_{O_2} values are obtained with stationary phase cells, when the carbohydrate content is 17–20% of the dry weight.

We have previously observed that aeration of washed suspensions of exponential phase cells rapidly reduces their carbohydrate content and Q_{O_2} value to low constant levels, and that ammonia is liberated into the supernatant after the carbohydrate had been metabolized (Dawes & Ribbons, 1962). It was concluded that the alkali-stable polyglucose (glycogen) serves initially as the endogenous substrate, and only after it has been utilized are nitrogenous materials oxidized with release of ammonia. Further support for this hypothesis is presented.

We have compared the endogenous respiratory activities of stationary phase cells obtained from glucose–ammonium salts, glucose–tryptone and tryptone media with respect to glycogen utilization and ammonia release. With cells from the media containing glucose, ammonia formation occurred only when the glycogen reserves had been depleted to negligible levels. In the case of tryptone-grown cells, ammonia was released immediately upon

incubation, but here the cells contained no glycogen.

It has been possible to correlate carbohydrate disappearance with the gaseous exchange. The oxygen consumed and carbon dioxide evolved during the initial stages of endogenous depletion of cells with high glycogen content were identical with those required for complete combustion of the utilized carbohydrate. Thereafter gaseous exchange exceeded that required by the utilization of glycogen, although disappearance of ribose (total orcinol-reactive material), presumably from degraded RNA, could account for one third of this excess.

Washed suspensions of aerobically or anaerobically grown cells held under anaerobic conditions displayed a similar rapid loss of glycogen reserves, which correlated well with the fall in $Q^{N_2}_{(CO_2+H_2)}$. Ammonia release began only after the glycogen was depleted. RNA was also degraded.

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Effects of Anoxia on the Metabolism of Amino Acids by the Adult Housefly (*Musca domestica*) *in vivo*

By G. M. Price and S. Moriya. (*Department of Biochemistry, Agricultural Research Council, Pest Infestation Laboratory, Slough, Bucks*)

Four-day-old houseflies were injected with [2-^{14}C]acetate and left in air for 1 hr. They were then divided into three groups. One group was killed immediately in liquid nitrogen (zero-time control), a second group was left in air for a further 2 hr. before being killed (aerobic flies) and a third group was put into O_2-free nitrogen for 2 hr. before being killed (anoxic flies). Extraction and radiochemical analysis of the free amino acids were carried out as previously described (Price, 1961*a*). Incorporation of ^{14}C-labelled amino acids into the 'protein' fraction of the fly was also determined as previously described (Price, 1961*a*).

In the aerobic flies the ^{14}C-activity of alanine, aspartate, glutamate, glutamine and proline fell by 65, 45, 60, 26 and 16%, respectively. The chemical level of alanine fell by 35%; however, there was little change in the chemical levels of the other amino acids. In the anoxic flies the ^{14}C-activity of alanine rose by 360%, while that of aspartate, glutamate, glutamine and proline fell by 84, 60, 84 and 32%, respectively. The chemical level of alanine rose by 400% while that of aspartate, glutamate and glutamine fell by 85, 20 and 55%, respectively.

Aspartate was depleted in the anoxic flies probably by transaminating with pyruvate to produce oxaloacetate and alanine. Since oxaloacetate does not accumulate in anoxic flies (Price, 1961*b*), it is possible that this compound is removed by combining with $NADH_2$ to produce malate and NAD. At least eight ^{14}C-labelled intermediates, other than free amino acids, were detectable in the ethanol extracts of anoxic flies. One of these has been identified chromatographically and by its disappearance on acid hydrolysis as glutathione. Four of the others have been identified chromatographically as fumarate, lactate, malate and succinate, respectively, the largest amount of ^{14}C-activity being found in succinate.

Specific radioactivity measurements of the protein amino acids showed that in the aerobic flies free ^{14}C-amino acids had been incorporated into protein over the 2 hr. period, whereas in the anoxic flies there had not been any incorporation.

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Perchloric Acid-Soluble Proteins

By D. Burston, M. P. Tombs and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

Previous work on the chromatographic fractionation of normal tissue and tumour proteins from autopsy material (Burston, Tombs & Maclagan, 1961) suggested that there was an increase in the level of the more acidic proteins in tumour and peritumour tissue as compared with normal tissue. The mixture was, however, too complex for a clear analysis of the α_1-globulin fraction to be made.

In order to simplify the extracts a method was developed for the isolation of glycoproteins from tissue extracts based on the perchloric acid method of Winzler, Devor, Mehl & Smyth (1948). In the case of normal liver extract a final concentration of 0·3N-perchloric acid gave an albumin-free filtrate containing a number of acidic and basic components.

All the tissues and tumours we have examined contained perchloric acid-soluble α_1-globulin, at least part of which was immunologically identical with the serum acid α_1-glycoprotein. Different tissues showed different general patterns, particularly in respect of the basic components. We have determined the levels of the various components in the filtrate by scanning the stained agar-gel electrophoresis slides, and have compared non-cancerous livers, livers with secondary tumours and non-tumorous livers from patients dying with tumours in other organs.

The liver α_1-globulin and acid mucopolysaccharide level was raised in all subjects with

tumours, irrespective of the tumour site. Attempts were made to isolate the various components of the 'normal' liver filtrate by first chromatographing on DEAE-cellulose to separate the basic from the acidic proteins and then chromatographing the basic fraction on carboxymethylcellulose with stepwise elution. The properties of six fractions obtained in this way will be discussed.

The α_1-glycoprotein was readily extracted from tissue by 0·3N-perchloric acid, but was accompanied by a number of basic tissue proteins not previously described.

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An Improved Method of Separating Amino Acids as *N*-2,4-Dinitrophenyl Derivatives: Determination of Valine

By N. A. MATHESON. (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Modifications of the method of Sanger (1945) for dinitrophenylating the free amino groups of peptides have been used for estimating individual amino acids in a mixture (e.g. Blackburn, 1949; Perrone, 1951; Mills, 1952; Callow & Work, 1952; Krol, 1952; Levy, 1954). Sanger (1945) originally isolated DNP-amino acids by a series of group separations using partition chromatography on silica gel.

It is commonly assumed that in the aqueous phase there is an equilibrium between ionized and non-ionized DNP-amino acid molecules but that only non-ionized molecules exist in the organic phase. Partition coefficients would then change by a factor of 2 for every change of 0·3 in the pH value of the buffer used, with related changes in *R* values. Perrone (1951) did find a strong pH dependence with ether, chloroform, etc. However, in the present work with ethyl acetate as the organic solvent, the *R* values of a number of DNP-derivatives were much less dependent on pH. These observations, with other evidence, implied that ionized DNP-amino acids were soluble in both phases and that partition of the ions was occurring. Khokhlov & Chi (1960) have noted that butanol extracts several DNP-amino acids even from 0·1N-NaOH, although they found the expected pH-dependence in butyl acetate-buffer systems.

On columns where partition of ions was occurring, DNP-amino acids gave unusually compact bands and, by varying salt concentration, pH or solvent, almost any one amino acid could be isolated from a mixture on a single column. For example, DNP-valine can be separated (*R*, approx. 0·5) by use of the two-phase mixture of 100 ml. of ethyl acetate with 20 ml. of 0·1M-tris chloride buffer (pH 8·2), containing 3·5% (w/v) NaCl. The bottom phase (2·5 ml.) is held on 4 g. of Hyflo Supercel (Johns-Manville) and development is carried out with the top phase. This separation, followed by spectrophotometric determination, gave satisfactory values for valine in synthetic mixtures and protein hydrolysates, provided the columns were carefully packed. The method is sensitive (0·1–1·5 μmole), accurate (range $\pm 4\%$ with valine), and rapid (DNP-valine takes 30 min. to pass through a column). Estimations on several samples can be run concurrently and no special apparatus is required.

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A Comparative Study of the Effects of Penicillin and Vancomycin

By P. E. REYNOLDS (introduced by E. F. GALE). (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Vancomycin and penicillin have similar effects on Gram-positive bacteria. Both antibiotics inhibit cell-wall synthesis in *Staphylococcus aureus*, but at bactericidal concentrations they do not affect nucleic acid or protein synthesis in washed suspensions of this organism. Park nucleotides (Park, 1952), believed to be precursors of cell-wall material (Strominger, Park & Thompson, 1959) accumulate in cells inhibited by either antibiotic.

Exponential cultures of *S. aureus* or *Bacillus megaterium* KM rapidly lost the ability to accumulate ^{14}C-labelled amino acids from the growth medium when treated with penicillin or vancomycin. The inhibition by penicillin of amino acid uptake in *S. aureus* was abolished when M-NaCl or M-NH_4Cl was included in the growth medium (Hancock, 1958). Cell-wall synthesis was still inhibited under these conditions, but the integrity of the protoplast membrane was maintained. The effects of vancomycin on cell-wall synthesis and amino-acid uptake were not reversed by M-NaCl or M-NH_4Cl.

The effect of penicillin on *B. megaterium* was more marked: 50% of the rods were converted into spheroplasts in the presence of a stabilizing concentration (10%) of sucrose. Under these conditions amino acid uptake continued though at a reduced rate. However, 10% sucrose did not protect the cells against vancomycin: the inhibition of amino acid uptake was not abolished and the culture lysed within a few hours.

Several workers (see review by Cooper, 1956) have reported an irreversible uptake of ^{35}S-labelled penicillin by bacteria. Pretreatment of a washed suspension of *S. aureus* at 0° with non-radioactive penicillin or bacitracin prevented uptake of radioactive penicillin in a subsequent incubation at 0°: treatment of the solution of radioactive penicillin with penicillinase also prevented uptake. Similar pretreatment with vancomycin or novobiocin did not reduce the uptake in the second incubation; neither did the simultaneous addition of vancomycin or novobiocin together with radioactive penicillin result in a reduced uptake. Substantially the same results were obtained in studies carried out with *B. megaterium*.

Penicillin, vancomycin, bacitracin and novobiocin can all cause the accumulation of Park nucleotides. The results of the above experiments suggest that bacitracin competes with penicillin for a site adjacent to, or the same as, the penicillin binding site, and that vancomycin has a different site of action.

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Metabolism of Phospholipids in the Rat

By J. D. Billimoria, Gwyneth Jones, B. F. Sansom* and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

Although much work has been done on the chemical aspects of the digestion and absorption of triglycerides, there is little information available on the metabolism of phospholipids during digestion.

Milk labelled with ^{32}P was obtained after injecting [^{32}P]orthophosphate into a goat. Two samples of milk at 6 and 20 hr. were obtained. Although the first milk specimen showed a higher activity per ml. than the second, the phospholipids in the latter had a higher specific activity.

Cream was obtained by centrifuging the milk and the ^{32}P was found to be equally distributed between the cream and the skimmed milk. Extraction of the lipids from the cream showed that although most of the label was concentrated in the casein approximately 2% of the ^{32}P was incorporated into the phospholipids.

The labelled phospholipids were subjected to a methanol–chloroform gradient-elution chromatography over silicic acid and for the present purpose the kephalin group of lipids (containing mainly ethanolamine and serine phosphatides) were separated from the choline lipids (lecithin and sphingomyelin) and each group separately fed to rats. Four hours after feeding the kephalin and choline lipids the animals were exsanguinated and the lipids were extracted from their blood and separated into kephalin, lecithin and sphingomyelin.

It was found that whether [^{32}P]kephalin or [^{32}P]-choline lipids were fed, the label in each case appeared to be distributed between the three main phospholipid groups of the blood. In each case the blood kephalin carried the smallest amount of the label, which was mainly found in the lecithin and sphingomyelin peaks.

These preliminary results indicate for the first time that during digestion the phospholipid fed undergoes cleavage at the phosphate-base bond and is then resynthesized. The possibility of complete breakdown to phosphate has not been excluded and is being further investigated.

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Investigation of Blood-Group Lipid Haptens

By O. W. Thiele and B. Urbaschek (introduced by Committee). (*Physiologisch-chemisches Institut der Universität, Göttingen, and Tierhygienisches Institut der Universität, Freiburg, Western Germany*)

The bovine blood group J is present in about 25% of all animals. The J substance is both attached to the erythrocytes and solved in the blood plasma (Ferguson, Stormont & Irvin, 1942). The total lipid extract from J-positive ox-serum shows hapten activity when tested in an haemolysis system (Urbaschek & Thiele, 1958). The total lipid was separated using acetone and ether successively. All hapten activity was found to be concentrated in the glycerol phosphatide fraction, all other fractions being immunologically negative. Column chromatography of the crude phosphatide yielded 18 fractions. One of them contained the J hapten. Its main constituent was sphingomyelin. Since the

activity disappears completely by treating with periodate and the ether extract of this fraction is more active than the ether-insoluble residue the J hapten was suggested to be an inositol phosphatide or a new substance other than glycolipid (Thiele, Wober & Urbaschek, 1962).

The total lipid prepared from human Rh-positive erythrocytes showed no specific hapten activity when tested *in vitro* using Rh antiserum in an agglutination test. We are therefore not able to confirm Carter's (1949) statement. On the other hand, the total lipid was able to lower *in vivo* both the Rh antibody contents of serum of rabbits previously immunized by human Rh-positive erythrocytes and the Rh antibody of human serum (Urbaschek, Thiele & Preisler, 1961). However, in most cases the lowering of Rh antibody contents was transient and did not suffice for the prevention of fetal erythroblastosis.

In another series of experiments we used crude lipopolysaccharide extracted from human erythrocytes by the procedure of Westphal, Lüderitz & Bister (1952). It was able to combine with Rh antibody *in vitro*, but was slightly antigenic when applied *in vivo*. The hapten activity was present both in lipopolysaccharide of Rh-positive and of rh-negative erythrocytes. Hence, we conclude the Rh receptor to be present in all sorts of human erythrocytes with the restriction that the receptor is not attached at the cell surface of rh-negative cells. After mild hydrolysis of lipopolysaccharide we succeeded in coating rh-negative cells which then behaved like Rh-positive cells. Coating of rh-negative cells was also possible when using lipopolysaccharide from rh-negative erythrocytes.

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Phospholipids of the Housefly (*Musca domestica*) Stable to Hydrolysis by Mild Alkali and Acid

By H. D. Crone and R. G. Bridges. (*Agricultural Research Council, Pest Infestation Laboratory, London Road, Slough, Bucks*)

In agreement with results reported by Bieber, Hodgson, Cheldelin, Brookes & Newburgh (1961) on the phospholipids of the blowfly *Phormia regina*, sphingomyelin was not found in phospholipid extracts of adult houseflies. However, when the hydrolysis procedures of Dawson (1960) were carried out on the housefly extracts, 4·6% of the phospholipid phosphorus remained lipid-bound after hydrolysis with mild alkali and mild acid. This 'stable fraction' was found to be eluted from a silicic acid column with, or just following, the kephalin fraction. It could be resolved into two components, both giving a positive reaction to ninhydrin, by chromatography on silicic acid impregnated paper, using the method of Marinetti & Stotz (1956). Hydrolysis of the 'stable fraction' with 15% (v/v) hydrochloric acid in methanol for 4 hr. at 100° rendered a further 3·5% of the total phospholipid phosphorus water soluble. Examination of the aqueous extract from this hydrolysis revealed the presence of ethanolamine phosphate, ethanolamine, a ninhydrin-positive phosphorus-containing compound and a ninhydrin-positive compound which had properties resembling those of sphingosine. Chromatography on silicic acid impregnated paper of the chloroform extract from the hydrochloric acid-methanol hydrolysis showed the presence of one major phosphorus-containing lipid, which no longer gave a positive ninhydrin test. The proportion of lipid-soluble to water-soluble phosphorus did not vary with hydrolysis times from 2 to 8 hr. The specific activities of the two fractions of this hydrolysate were different when extracts of phospholipids from flies fed on [^{32}P]orthophosphate were examined.

These results are consistent with the presence of two compounds in this 'stable fraction'. The fact that after methanolic HCl hydrolysis ethanolamine is detected and a fraction of the phosphorus remains lipid bound suggests the presence of a glycerol–ether phospholipid containing ethanolamine of the type described by Carter, Smith & Jones (1958) from egg yolk and by Ansell & Spanner (1961) from mammalian brain. The presence of ethanolamine phosphate, a sphingosine-like compound (no cerebrosides were detected in the extracts) and a phosphorus-containing ninhydrin-positive compound (possibly sphingosylphosphorylethanolamine) strongly indicates the occurrence of a sphingomyelin type compound in which ethanolamine replaces choline. It is interesting to note that phosphatidylethanolamine is the major phospholipid of houseflies, accounting for 65% of the lipid phosphorus.

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The Contribution of Glucose and Acetate to Milk Constituents and Carbon Dioxide in the Isolated Perfused Udder

By D. C. HARDWICK and J. L. LINZELL. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

In agreement with much previous work on ruminants we have shown that the production of milk fat in the isolated perfused goat udder is dependent on acetate (Hardwick, Linzell & Price, 1961). This accords with the suggestion of Glascock (1958) that 'the contribution of carbohydrate to the C_2 pool is very small in ruminants'. We have now investigated the distribution of ^{14}C in milk from glands given both glucose and acetate continuously after the second hour (together with amino acids and minerals) but with either [U-^{14}C]glucose or [$^{14}C_2$]acetate.

In two experiments with labelled glucose, milk lactose, milk-triglyceride glycerol and respired CO_2 had high specific activity as expected. But, whereas the fatty acids of milk triglycerides were not significantly labelled during the 6 hr. after starting the infusion milk citrate was labelled within 1 hr. and the final tissue citrate had a similar specific activity to that in the last milk sample obtained. About 30 % of the citrate came from glucose.

The milk lactose and the milk-triglyceride glycerol during the experiment with the infusion of [$^{14}C_2$]-acetate were only slightly labelled. Carbon dioxide had a fairly low specific activity, but milk citrate was very active and about 30 % of the citrate came from acetate. The fatty acids of the milk triglycerides also had a high activity, the non-volatile acids being almost as strongly labelled as the complete mixture of acids.

If we assume that milk citrate is synthesized by the Krebs cycle, glucose must contribute to it either via acetyl-CoA or via oxaloacetate. If via acetyl-CoA, this does not appear to be available for the fatty acid synthesis, suggesting two acetyl-CoA pools. If via oxaloacetate, then pyruvate derived from glucose does not form acetyl-CoA readily in the presence of acetate.

These results also confirm that the ruminant mammary gland receiving glucose and acetate in about the same proportion as in the conscious animal (3:1 by weight, 1:1 molar ratio) oxidizes both, for under the conditions of our experiments about 10 % of the glucose and 3·5 % of the acetate were metabolized to CO_2; at least 45 % of CO_2 comes directly from glucose infused and 10 % from acetate infused.

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Regularities of Group Retention Factors in Gas–Liquid Chromatography of Steroids

By C. J. W. BROOKS and L. HANAINEH. (*Medical Research Council Clinical Chemotherapy Research Unit, Western Infirmary, Glasgow*)

Clayton (1961, 1962) has shown that the retention time of a polyfunctional steroid may be evaluated, in suitable cases, by compounding 'group retention factors', characteristic of each substituent, with the retention time of the unsubstituted steroid. The analytical value of such correlations was emphasized. Group retention factors may be regarded as the counterpart of the R_M values (Bush, 1961) of paper chromatography.

Clayton used a polyester stationary phase and studied principally sterol methyl ethers. The present work deals mainly with C_{19} and C_{21} steroids, and SE-30 has been used as an almost non-selective phase of high thermal stability, affording the simplest retention behaviour which is moreover reproducible in various laboratories. All data have been obtained with the Pye Argon Chromatograph, at 200° except as stated. Retention times are expressed relative to cholestane and are independent of flow rate.

A preliminary study of vicinal interactions showed that the retention factor for the 3β-hydroxyl group (1·98 at 225°) was unaffected by the presence of unsaturation at $C_{(5)}$ or a ketone at $C_{(6)}$. On the other hand, our present results suggest that this factor is not wholly independent of the nature of the side-chain at $C_{(17)}$. For a given nuclear type (C_{19} or C_{21}) reasonably constant factors may be assigned for the introduction of hydroxyl or ketone groups at positions 3, 11 and 17 (or 20).

Steric effects are illustrated by (i) the sequence of retention factors for ketones at positions 3 (2·2–2·3), 17 (2·0), 20 (1·9) and 11 (1·3–1·4); (ii) the regular elution of 5β-steroids before their 5α-isomers (nine examples) with a separation factor of 1·12 (S.D. 0·05) (cf. VandenHeuvel, Sjövall & Horning, 1961; separation factor 1·13 at 210°).

Further work is in progress on the scope of these regularities.

We thank Professor E. C. Horning for his encouragement, and Professor W. Klyne for gifts of samples from the Medical Research Council Steroid Reference Collection.

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Separation of Protein and Nucleic Acid by Zone Electrophoresis in Phenol–Acetic Acid–Water

By M. Bagdasarian, N. A. Matheson, R. L. M. Synge and Mary A. Youngson. (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Proteins, on filter-paper electrophoresis in phenol–acetic acid–water (1:1:1, w:v:v), have rather uniform cationic mobilities which vary with the content of basic amino acid residues less than would be predicted from simple theory (Synge, 1957). With mixtures in this solvent of commercial preparations of yeast RNA and horse-heart cytochrome *c* (both 1 %, w/v) electrophoresis gave ready separation. RNA, in the absence of protein, remains on the starting line, as does *N*-(2, 4-dinitrophenyl)-ethanolamine, the marker for electroendosmosis. With the mixture, there is initially interaction resulting in slight cationic migration of RNA, but the components soon separate, with slight 'tails' pointing towards one another. Tailing does not increase as electrophoresis proceeds. The interaction is thus very much less than those reported in mainly aqueous electrolytes (e.g. Eliasson, Hammarsten, Lindahl & Palmstierna, 1961), presumably by virtue of the power of the solvent mixture to disrupt hydrogen bonds.

We have made extracts of broad-bean leaves with this solvent mixture; these contain 90 % or more of the leaf protein. We have fractionated these extracts by dialysis or by chromatography on Sephadex G-75 (AB Pharmacia, Uppsala) in the same solvent mixture (cf. Synge & Youngson, 1961). We thus obtain high-molecular fractions. On column electrophoresis (Haglund-Tiselius) of these fractions in the same solvent mixture, most of the protein migrates cationically as a fairly compact zone. Materials of markedly different amino acid composition occur in zones migrating both faster and slower than this. We are also studying the distribution of lipid, carbohydrate and nucleic acid components in the various fractions. The low conductivity of the electrolyte mixture, its freedom from N and its volatility make it convenient for preparative work.

O-Polypeptidyl derivatives of 'soluble' RNA have been postulated as intermediates in protein biosynthesis (Lipmann, 1961). The electrophoretic system here described might prove useful for studying their possible occurrence, since in it they would be expected to have cationic mobilities markedly less than those of ordinary proteins.

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The Titration Properties of High-Molecular-Weight Ribonucleic Acid from Rat-Liver and Model Polynucleotides

By R. A. Cox. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The dissociation properties of polynucleotides reflect their secondary structure (e.g. Gulland, Jordan & Taylor, 1947; Steiner & Beers, 1959) and for this reason ribosomal, viral and soluble RNA were studied. The dissociation curves of high-molecular-weight RNA from rat liver typify the results obtained. RNA from rat liver (Laskov, Margoliash, Littauer & Eisenberg, 1959) was titrated with acid and alkali at 0·4°. The dissociation curve (I) obtained on titration from pH 7 to pH 3 was different from the curve (II) obtained on titration from pH 3–7. Curves I and II were accurately repeated on subsequent cycles. About 50 % of the titratable groups dissociated over the range pH 7–11·6.

DNA from calf thymus (Kay, Simmons & Dounce, 1952) was titrated at 25°. Few, if any, of the titratable groups were ionized at pH 11·4 but ionization was complete at pH 11·6 (curve I). A broader curve (II) which was reversible over the range pH 3–11·6 was obtained on titration with acid from pH 11·6. About half the groups of a partly denatured sample ionized reversibly over the range pH 8–11·4 and half over the range pH 11·4–11·6.

The complex poly (A + U) formed between polyadenylic acid (poly A) and polyuridylic acid (poly U) at pH 7 was titrated spectrophotometrically. Dissociation into its component polynucleotides took place over the ranges pH 3–4·5 and pH 10·25–10·75 at 22° and pH 4–5·5 and pH 9·25–10·0 at 39·4°. In alkaline solutions the interaction between poly A and poly U was reversible (cf. Steiner & Beers, 1959). Hysteresis was found in acidic solutions since poly (A + U) reformed over the range pH 5·1–6·0 at 22° and pH 4·5–5·7 at 39·4°. Irreversible changes were noticed when poly (A + U), initially at pH 7 was brought to acidic pH values. At pH 4·60 poly (A + U) dissociated irreversibly into its components upon heating to 55°. Poly A and poly U did not interact on mixing at pH 5·05 at 22° (cf. Steiner & Beers, 1959) but a complex was formed upon cooling slowly from 80°.

It was noticed that both poly (A + U) and the acid form of poly A were stable over the range pH 4–5·5 at 22°. In contrast to poly (A + U) the stability of the acid form of poly A increased on

acidification and was stable at 80° at pH 4·0. Hysteresis therefore arises because two ordered polynucleotide conformations are both stable over a particular range of pH value and because thermodynamic equilibrium is not attained.

These studies provide further evidence that RNA has a partly helical conformation at neutral pH (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959) that breaks down reversibly on titration with alkali. The hysteresis found on titration with acid may be explained by analogy with poly (A+U) if an ordered form of RNA corresponding to the acid form of poly A which is stable at low pH were formed on disruption of ordered domains stable at pH 7; if both ordered forms were stable at pH values within the range where curves I and II differed; and if equilibrium were not attained.

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Some Aspects of the Use of Spectrophotometric Data for the Evaluation of the Melting Temperature of the Double-Stranded Helix of Polynucleotides

By S. Lewin. (*Physical-Biochemistry Research School, South-West Essex Technical College, London, E.* 17)

The dissociation of double-stranded helices of polynucleotides into their constituent strands is known to be associated with increase in extinction and other physical properties and may be characterized by the melting temperature T_m (for references see Doty, 1962).

Published work on the variations of extinction with temperature refers usually to a specified wavelength in the region of 258 to 260 mμ where the peaks of the absorption curves of DNA, RNA and other polynucleotides are normally found.

Work in this laboratory has shown that while some polynucleotide interactions which are accompanied by opening of the double-stranded helix (e.g. DNA with urea) are accompanied only by an overall increase in extinction, in which the effect is more predominant at the peak of the curve region than at the crest region $\sim$ 232 mμ, other reactions (e.g. DNA or RNA with formaldehyde) are accompanied also by a lateral displacement of the curve to the region of higher wavelengths. As both of the above classes of reactions are associated with dissociation of the double-stranded helices of the polynucleotides present, it was considered pertinent to investigate the variation of the light absorption curves of salmon sperm DNA with temperature, over the range of 30° to 95°. It was found that under certain experimental conditions, e.g. those involving 0·02 g. of DNA/l. in ionic strengths of 10^{-3} to 10^{-2} in acetate buffers, pH 4·7, the increase in extinction in the transition temperature region was significantly greater in the trough wavelength region than in the corresponding crest wavelength region; and that some lateral displacement could be noted.

A similar, but not as pronounced, effect was noted in the variation of the light absorption curve of calf-thymus DNA on change of pH value from 7·0 to 12·3, when the change in extinction at the trough was somewhat greater than that at the peak (Shack & Thompsett, 1952).

The greater sensitivity of the trough region to extinction changes, under certain conditions, suggests the possibility of its use in the evaluation of T_m, particularly where the high stability of the double-stranded helix may require extrapolation to values above 100°.

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The Luminescence of Purines and Pyrimidines

By J. W. Longworth (introduced by G. Weber). (*Department of Biochemistry, University of Sheffield*)

A fluorescence of small yield can be detected from several purines, when they are dissolved in a polar glass (50% propane-1,2-diol–50% 0·01M-phosphate buffer, pH 7·0) at 77° K. The fluorescence maxima for the derivatives purine, adenine, guanine and caffeine are at 297, (293, 306), 323 and 317 mμ respectively, with a fluorescence yield (q_f) of 0·02, 0·06, 0·09 and 0·10 respectively. The fluorescence of adenine is characterized by the presence of vibrational structure in contrast to the other purines. A characteristically structured phosphorescence can also be found, with the emission lying in the spectral range 360–500 mμ, and the respective main maxima at 400, 388, 410 and 412 mμ, while the ratio of the quantum yield of phosphorescence (q_p) and fluorescence has values of 1·9, 0·75, 0·4 and 0·2 respectively. Again the emission of adenine possesses much structure and occurs to shorter wavelengths than the other purine derivatives. The phosphorescence spectra of adenine and guanine differ for the several ionic states that the compounds possess.

A luminescence can also be measured from the nucleoside and nucleotide derivatives of adenine and guanine, but they have lower quantum yields than the parent bases. The spectral location of the guanine derivatives differ little from that of guanine, guanosine and guanylic acid have a fluorescence maximum at 320 mμ, and the main phosphorescence maximum at 397 mμ and the yield ratio (q_f/q_p) of 0·9 and 0·5 respectively. Larger differences occur for the adenine compounds, with emission maxima at 320 and 402 mμ for adenosine and 312 and 400 mμ for adenylic acid, with yield ratio of 1·0 and 0·9 respectively. A very weak phosphorescence has also been detected with deoxyribonucleic acid in the same spectral range as the nucleotides.

To further characterize the emission process, and to confirm that the luminescence is an intrinsic property of the purines, the excitation spectra of the fluorescence and the phosphorescence have been determined. The spectra are found to be essentially similar to the room temperature absorption spectra, and so eliminates the possibility of impurities causing the emissions.

Purine is non-fluorescent in hydrocarbon solvents and the absorption spectrum has a weak band to longer wavelengths than the long wavelength edge of the absorption in polar solvents. This implies that an n–π transition occurs on the long wavelength edge of the absorption (Kasha, 1961) and the behaviour can be found with several heterocyclic bases. The absorption polarization spectrum of the fluorescence for several purines can be interpreted on the basis of three transitions occurring within the single envelope of the last absorption band, in agreement with the absorption spectral data of Mason (1954), if an n–π transition is also included.

The naturally occurring pyrimidines (uracil, thymine, cytosine) have been found to be non-fluorescent or phosphorescent in polar solvent at 77° K, though weak luminescences can be detected in hydrocarbon solvents. 2-Aminopyrimidine is fluorescent at room temperatures (q_f 0·12, max. 353 mμ in water) and has a strong phosphorescence (max. 387 mμ, q_p 0·18, q_p/q_f 1·2). The fluorescence absorption polarization spectrum can also be interpreted by three transitions within the last absorption band (Mason, 1959).

I wish to thank Dr S. Ainsworth for making his recording fluorescence polarization apparatus freely available, and the Medical Research Council for a scholarship.

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Utilization of Glucose in the Rat Brain

By M. K. Gaitonde, D. Richter and R. Vrba. (*Neuropsychiatric Research Unit, Medical Research Council, Woodmansterne Road, Carshalton, Surrey*)

Experiments with labelled glucose have indicated that under normal conditions *in vivo* only a part of the glucose taken up by the brain from the blood is oxidized directly to CO_2. As much as one-half of the glucose carbon is incorporated into tissue constituents which are subsequently utilized as a source of energy (Sacks, 1957, 1961). Similar findings have been reported for the perfused cat brain, in which about 70 % of the respiratory CO_2 is apparently derived from non-carbohydrate sources (Allweis & Magnes, 1958; Geiger, Kawakita & Barkulis, 1960).

The fate of the glucose taken up by the brain and not oxidized to CO_2 has not been clearly defined, but a part is known to be incorporated into amino acids, proteins, lipids and other compounds (Roberts, Flexner & Flexner, 1959). In the present experiments the distribution of ^{14}C was determined in rat organs at different times after injection of [U-^{14}C]glucose: the experimental methods and conditions are described elsewhere (Vrba, Gaitonde & Richter, 1962). During the first 30 min. the ^{14}C content of the brain (counts/min./g. of fresh tissue) rapidly increased to several times that in liver, kidney, heart, spleen, muscle, lung or blood. At 30 min. after injection 0·7 % of the total ^{14}C retained in the brain was in the lipids, 0·4 % in the proteins, 0·5 % in nucleic acid and 98·3 % in acid-soluble metabolites. Of the total ^{14}C in the brain acid-soluble fraction at 30 min. after injection, 2 % was in neutral compounds including glucose, 4 % in carboxylic acids and 94 % was combined in amino acids. The specific radioactivity of the brain amino acid fraction, which was far higher than that in any other organ examined, was mainly due to glutamic and aspartic acids. The specific radioactivities (counts/min./mg. α-NH_2) at 30 min. of aspartic and glutamic acids respectively in different organs were: brain 27 400 and 76 300; liver 9100 and 5720; kidney 3480 and 5710; heart 4550 and 5340; skeletal muscle 3250 and 1350; lung 650 and 2520; blood 1235 and 1765.

The ^{14}C-content of the brain amino acid fraction decreased as the labelled amino acids were metabolized or incorporated into protein. Since there is no net loss of amino acids from the brain to the blood, these observations give evidence that the formation and oxidation of amino acids, which occurs in all organs to some extent, constitutes a main pathway of glucose metabolism in the brain.

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Relationship of Acute Ammonia-Intoxication Effects to Brain and Blood Ammonia Levels in Rats

By V. Bocchini, F. Salvatore and F. Cimino (introduced by Committee). (*Institute of Biological Chemistry, University of Naples, Naples, Italy*)

In previous researches (Salvatore & Bocchini, 1961) it was shown that in rats intraperitoneally given LD_{50} doses of ammonium acetate, the blood ammonia levels were strongly different at 15 and 60 min. after the injection. However, the decrease in blood ammonia levels did not always parallel the alleviation of toxic symptomatology (convulsions, coma, etc.). Since the evidence for a relationship between convulsive disorders and brain and blood ammonia content (Torda, 1953; Du Ruisseau, Greenstein, Winitz & Birnbaum, 1957; Navioaz, Gerritsen & Wright, 1961) is not conclusive, it appeared interesting to compare the changes in brain ammonia levels with those occurring in the blood at different intervals from the injection.

Male rats of the Long-Evans strain weighing 250–300 g. and fasted for 12 hr. were used in these experiments. Ammonium acetate was injected intraperitoneally in the dose of $8{\cdot}2 \pm 0{\cdot}8$ m-moles/kg. (LD_{50}). In survivors, blood samples were drawn by cardiac puncture at 5 min. intervals from 5 to 60 min. after the intoxication. Separate rats treated in the same way were killed in liquid nitrogen at the same intervals as before and the brain was removed. Blood and brain ammonia estimation was performed according to Seligson & Hirahara (1957).

A sharp increase of blood ammonia levels was observed in the first 5 min. and after a slight decrease a peak was reached after 20 min.; afterwards it gradually decreased. A different pattern occurred in the brain: free ammonia increased in the first 25 min., its level remained approximately unchanged until 50 min., afterwards a slow decrease was observed. In equally intoxicated rats a Gauss-type curve was constructed on the basis of the incidence of mortality during the time. The highest incidence of mortality occurred 25 min. after intoxication with the ammonium salt; the incomplete agreement of this time with that of the highest ammonia level in blood is noteworthy. On the other hand, toxic symptoms were observed to be related to ammonia levels in the brain better than to those in the blood. It is possible, therefore, that changes in the metabolic properties of the central nervous system, directly produced by ammonia, are responsible for the toxic symptoms. A short discussion of our results and those of other authors will be presented.

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An Abnormality of Citrulline Metabolism in a Mentally Retarded Child

By W. C. McMurray, F. Mohyuddin, R. J. Rossiter, J. C. Rathbun and D. E. Zarfas. (*Department of Biochemistry and Department of Paediatrics, University of Western Ontario and Children's Psychiatric Research Institute, London, Ontario, Canada*)

An amino acid present in large quantities in the urine of a mentally retarded child has been isolated and identified as L-citrulline (McMurray *et al.* 1962). On the basis of the high concentrations of citrulline found in the body fluids of the patient the defect appears to be of metabolic rather than of renal origin. It has been suggested that the condition arises from a metabolic block in the utilization of citrulline, although the finding of normal concentrations of urea in the patient's blood and urine makes it appear unlikely that the conversion of citrulline into argininosuccinic acid by the liver is the site of the lesion.

When the patient's protein intake was increased or decreased, the urinary excretion of both citrulline and urea changed in the same direction. This probably explains the previously reported variability in the daily excretion of citrulline (0·5–3 g./day). The concentration of citrulline in the blood remained relatively constant. An oral load of citrulline or ornithine caused an increase in the concentration of citrulline in both blood and urine. These findings and results of loading studies with other amino acids will be discussed.

These studies were aided by a grant from the Junior Red Cross of Ontario.

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Partial Purification of a Cerebral Phosphoprotein Fraction

By S. P. R. Rose.* (*Department of Biochemistry, South Parks Road, Oxford*)

Phosphoprotein has hitherto been assayed on the basis of methods described by Schmidt & Thannhauser (1945) and Schneider (1945, 1946). Using these methods, Heald (1961) prepared a phosphoprotein fraction from brain about fourfold purified in terms of nitrogen. The finding of an enzyme in brain specifically releasing phosphate from casein and phosvitin (Rose & Heald, 1961) suggested its use as an enzymic assay for the endogenous tissue phosphoproteins. The phosphoprotein samples were prepared by dispersing the tissue (80 mg. or more) in water or 1·0N-NaOH and dialysing overnight against 200 vol. of glass-distilled water to remove low-molecular-weight phosphates. The dispersion was then incubated for 30 min. at pH 5·5 and 37° with purified brain phosphoprotein phosphatase and the phosphate released determined. The release of phosphate was proportional to substrate, and, in the presence of sufficient enzyme, was complete within 15 min. It was equivalent to $1{\cdot}13 \pm 0{\cdot}06$ μmole enzymically-labile P/g. of guinea-pig brain. This compares with the figure of 1·1 μmole alkali-labile P/g. found by Heald (1960). The enzyme thus quantitatively released the phosphate of cerebral phosphoprotein. It was found to act similarly on the phosphoproteins of guinea-pig kidney, liver and gut, and human red cells. Tissue preparations made by rapid dispersion in NaOH gave higher phosphoprotein values than those made in water.

With this assay, a partial purification of the phosphoproteins of whole ox brain has been obtained. The brain was dispersed with 3 vol. of 1·0N-NaOH, centrifuged, and the residue extracted with a further 2 vol. of NaOH and centrifuged again. The pH of the combined supernatants was lowered to 7·2–7·3 with 5M-acetic acid and the suspension centrifuged. That fraction of the supernatant which precipitated between 55% and 90 (v/v) ethanol was collected, dissolved in water, and dialysed. The resulting solution represented a 40–50% yield of a 15- to 25-fold purified phosphoprotein fraction. Further purification steps are under investigation.

I am grateful to Professor H. A. Krebs for helpful discussions.

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Nucleotide Sequences in Purified Soluble-Ribonucleic Acid Fractions

By M. Staehelin, H.-G. Zachau and M. Schweiger (introduced by Committee). (*Research Laboratories of the Pharmaceutical Department of CIBA Limited, Basle, Switzerland, and Institut für Genetik der Universität, Köln, Germany*)

In an attempt to gain more insight in the structure of s-RNA the frequency in which some base sequences occurred in various s-RNA fractions was determined.

Purified fractions of s-RNA in which the amino acid-incorporating activities for valine (valine fraction) and serine (serine fraction) were enriched were digested with pancreatic ribonuclease. The components of the digest were separated by chromatography on DEAE-cellulose. Adenosine was found to correspond to 1·25% of the total nucleotides. Cytidylic acid was found to be the main mononucleotide liberated. On the basis of eighty nucleotides per chain in total s-RNA as well as in the valine fraction six residues of uridylic acid and two residues of pseudouridylic acid were liberated. In serine s-RNA eight residues of uridylic acid and practically no pseudouridylic acid were found.

Two additional dinucleotides containing both 2-dimethylguanine, and cytidylic acid and pseudouridylic acid respectively, were found in total s-RNA and in the valine fraction but not in the serine fraction. Normal dinucleotides were liberated to a markedly different extent from the various fractions. In the total s-RNA the ratio of ApCp:ApUp:GpCp:GpUp was roughly 2:1:3:2. In the valine fraction this ratio approached a value of 1:1:1:1, whereas in the serine fraction a ratio of about 0:1:5:2 was observed. This fraction also contained a residue of an additional dinucleotide which is a derivative of GpCp. The distribution of normal and additional tri- and tetra-nucleotides will also be reported.

Bacterial Ribonucleoprotein Particles which Sediment more slowly than Ribosomes

By G. Turnock, A. E. White and D. G. Wild. (*Department of Biochemistry, University of Leeds, Leeds 2*)

Although most of the ribonucleoprotein (RNP) of bacteria is normally found in 30 s and 50 s ribo-

somes, RNP-particles which sediment more slowly, at 14–18 s, are observed when growth of *Escherichia coli* is inhibited by chloramphenicol (Dagley & Sykes, 1959; Nomura & Watson, 1959) or by puromycin (Dagley, Sykes, White & Wild, 1962) and also when a certain methionine-requiring mutant is starved of this amino acid (Dagley *et al.* 1962). The particles from these different systems are also more sensitive to ribonuclease and sonic vibrations than are ribosomes; and when magnesium ions are added, the particles sediment faster and give rise to a broad boundary at about 30 s.

We have studied certain differences between particles from the different sources. It is well established (Neidhardt & Gros, 1957; Hahn, Schaechter, Cezlowski, Hopps & Ciak, 1957), and we have confirmed, that a large part of the chloramphenicol-RNA (CM-RNA) is degraded when the cells are washed free from antibiotic and incubated in buffer. The CM-RNP particles are lost during this incubation. On the other hand, both puromycin- and mutant-RNA are stable. CM-RNP particles contain less protein and more RNA than do normal ribosomes (Nomura & Watson, 1959; Dagley & Sykes, 1960), whereas those formed by the mutant have the same ratio of RNA to protein as 50 s ribosomes. Experiments with [^{35}S]sulphate and [^{35}S]methionine show that the protein moiety of this mutant-RNP resembles that of 50 s ribosomes in its content of sulphur-containing amino acids.

Incubation with antibiotic may inhibit protein synthesis and permit accumulation of RNA without the formation of 14–18 s particles: this is so for a strain of *Bacillus megaterium* incubated with chloramphenicol or erythromycin and for *E. coli* incubated with puromycin in a mineral salts medium instead of one containing peptone.

The study of these RNP-particles has been extended by the discovery that when 5-methyltryptophan is added to the methionine-requiring mutant, growth and protein synthesis are inhibited but considerable RNA synthesis takes place. RNP-particles sedimenting at about 16 s again accumulate. Changes in this RNP have been followed during the time elapsing between addition of tryptophan and resumption of growth. The sedimentation coefficient of the particles appears to increase so that they sediment close to the 30 s ribosomes; simultaneously an additional small boundary sedimenting at about 40 s becomes visible. Both these 'new' species are as sensitive to RNA-ase as the original 16 s particles.

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The Role of a Large-Particle Fraction from Yeast in Protein Synthesis

By J. G. Hauge and H. O. Halvorson (introduced by Committee). (*National Institute of Public Health, Oslo, Norway and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, U.S.A.*)

Ribosomes attached to lipoprotein membranes are the most active sites for protein synthesis in mammalian cells (Siekevitz & Pallade, 1960) and in bacteria (McQuillen, Roberts & Britten, 1959; Butler, Crathorn & Hunter, 1958). The membrane is in the former instance the endoplasmic reticulum, in the latter the cytoplasmic membrane. A system for incorporation of amino acids into proteins has furthermore been found in an RNA-enriched subunit of mammalian mitochondria (Roodyn, Reis & Work, 1961). Yeast is the simplest organism in which both an endoplasmic reticulum and mitochondria are known to exist, possibly in intimate connexion with each other (Thyagarajan, Conti & Naylor, 1961). It was of interest therefore to determine whether this internal membrane system is the counterpart of the cytoplasmic membrane of bacterial cells and plays an active role in protein synthesis. Previous studies *in vivo* had shown that free ribosomes in yeast were labelled several times faster than soluble proteins (Young, Kihara & Halvorson, 1961).

In the present study the larger particles from French pressure cell homogenates of the hybrid *Saccharomyces fragilis* × *Saccharomyces dobzanskii* were isolated after short exposures to radioactive sulphate. The particles were found to become labelled at a rate several times that of the free ribosomal particles isolated in the same experiments. Over half of this newly incorporated radioactivity was displaced within 10 sec. upon addition of unlabelled sulphur amino acids. Nascent protein, therefore, seems to be associated with the fraction. Radioautographs of two-dimensional chromatograms of partial and complete hydrolysates of the particles showed the radioactivity to be present exclusively as sulphur amino acids in peptide linkages.

Relatively high levels of electron transport enzymes and carriers were found associated with the particles, indicating material of mitochondrial origin. The chemical composition of a typical preparation was 52% protein, 27% lipid and 21% RNA. The surprisingly high amount of RNA present could not be reduced by procedures that would

remove free ribosomes. It appears, at least partly, to represent membrane-bound ribosomes. In electron microscopic thin sections of particle preparations, sections of numerous vesicles or tubes appear, many of which can be seen to have granules of the size of yeast ribosomes attached.

The evidence suggests the participation in protein synthesis in yeast of ribosomes bound to mitochondria or an endoplasmic reticulum, or to a complex of the two.

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Immunoelectrophoretic and Radioautographic Studies of Rat Serum Albumin after Incorporation of ^{14}C-labelled Amino Acids *in vitro*

By Alexandra von der Decken. (*The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden*)

Ribonucleoprotein particles prepared by treatment of a rat-liver homogenate with deoxycholate, Lubrol-W and concentrated KCl (Rendi & Hultin, 1960) show an apparent incorporation of [^{14}C]amino acids into rat serum albumin when incubated with cell sap and a source of ATP (von der Decken & Campbell, 1962). However, when purified by zone electrophoresis most of the radioactivity of the albumin is lost in contrast to what is observed when whole microsomes were used (Campbell, Greengard & Kernot, 1960).

In the present investigation, the possible formation of serum albumin by the particles and some characteristic properties of the product were further studied by use of immunoelectrophoresis and autoradiography. Ribonucleoprotein particles from 30 to 40 g. of liver were suspended in a tris-buffered medium. They were incubated for 2–3 hr. at 35° with 2 ml. of a 105 000*g* supernatant (cell sap) from a 30% rat-liver homogenate in the presence of ATP, phosphoenol pyruvate, pyruvate kinase and [^{14}C]leucine. After incubation, 2 ml. of chick-liver cell sap were added. The particles were separated by centrifugation for 1 hr. at 105 000*g*, subjected to ultrasonic vibrations and again centrifuged. The soluble fractions were combined, freeze-dried and dissolved in a minimum amount of water. The solution was applied to agar electrophoresis, 0·4 mA/cm. cross-section (agar layer 2 mm. high) for 21 hr. Antisera specific for either rat serum albumin or chick serum albumin were added.

With these antisera, precipitates corresponding to either rat or chick serum albumin appeared after immunoelectrophoresis. However, there also appeared a number of precipitates with antigens of lower electrophoretic mobilities than the albumins. Most of these antigens seemed to be partially identical immunologically with the corresponding albumins. After treatment of the fractions with ribonuclease (20 μg./ml.) the number of precipitates corresponding to antigens of irregular mobilities was reduced. This was also the case if the extracts were dialysed before electrophoresis. It seems likely that these precipitates were formed by albumin attached to ribosomal fragments or RNA.

Upon radioautography (exposure time 1–2 weeks) of the agar plates obtained with the untreated extracts no clear-cut labelling could be observed of the immunological precipitates. However, these radioautographs showed a strong blackening due to the presence of material which did not react with the antisera. Most of this material disappeared upon treatment of the fractions with ribonuclease. In the radioautographs made with these treated fractions a strong labelling of the rat albumin precipitate appeared. This labelling was specific since the precipitate of the chick albumin remained unlabelled. A labelling of the rat albumin was also seen after dialysis of the extracts. Purification of the albumin by means of chromatography on DEAE-cellulose reduced the labelling without, however, abolishing it completely. The nature and distribution of this labelling are presently under investigation.

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Inactivation of Ribosomes by Antrycide

By B. A. Newton. (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The trypanocidal drug antrycide (4,2′-diamino-2,6′-dimethyl-6,4′-pyrimidylaminoquinoline 1,1′-dimethosulphate) rapidly inhibits [^{14}C]purine incorporation into ribonucleic acid of the flagellate *Strigomonas oncopelti* (Newton, 1958). The drug does not affect the permeability of *S. oncopelti* to

[^{14}C]adenine nor does it inhibit the conversion of this purine into nucleoside or nucleotides (Newton, 1960). These results suggest that antrycide inhibits some stage in the polymerization of acid-soluble nucleotides to nucleic acid. Polynucleotide phosphorylase isolated from *S. oncopelti* was not inactivated by antrycide at a concentration which inhibited adenine incorporation by 80%. It has now been established that [^{14}C]adenine incorporation by this organism is a measure of nucleic acid synthesis and that this nucleic acid contains bases in the normal ratios: it does not represent 'end-group labelling' or the synthesis of polyadenylic acid.

A study of [^{14}C]adenine distribution in fractions of disrupted organisms has shown that the drug inhibits the incorporation of this purine into ribosomes to a greater extent than into any other cell fraction: incorporation into soluble RNA was initially unaffected.

Growth of *S. oncopelti* is not completely inhibited by antrycide, but the pattern of growth is changed from a logarithmic form to a linear form; in the course of growth in the presence of drug a progressive decrease in the 80 s ribosome fraction of organisms has been observed. Analysis of cell fractions has revealed that this decrease is due to an aggregation of ribosomal material which, consequently, was sedimented by low-speed centrifugation. More than 90% of the ribosomes were found to be aggregated after 8 hr. growth in the presence of drug. A positive correlation between the amount of 80 s ribosomal material in antrycide-grown organisms and the rate at which these organisms incorporate [^{14}C]amino acids has been demonstrated, suggesting that aggregation of ribosomes also results in their inactivation.

Transfer of organisms from a drug containing medium to a drug-free medium is accompanied by a return to a logarithmic form of growth. Evidence will be presented to show that this recovery is associated with a synthesis of new 80 s ribosomes and not with a disaggregation of drug-inactivated ribosomes.

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The Inhibition of Protein Synthesis by Chlortetracycline in Cell-Free Systems

By T. J. Franklin. (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

Chlortetracycline has been known to inhibit protein synthesis in bacterial cells for a number of years (Gale & Folkes, 1953). More recently, it has been shown to depress the incorporation of methionine into rabbit tissue proteins *in vivo* (Nikolov & Ilkov, 1961). Little, however, has been done to determine its site of action in the protein biosynthetic pathway. In this investigation the effect of chlortetracycline on the incorporation *in vitro* of L-[^{14}C]leucine into the various fractions of cell-free preparations from rat liver has been studied. The incorporation of the amino acid into the whole protein of a suspension of microsomes in cell sap was considerably inhibited by chlortetracycline. The drug was found to have no effect on the extent of incorporation of the amino acid into the transfer RNA when the pH 5·0 fraction was incubated with chlortetracycline, L-[^{14}C]leucine and ATP. When, however, a microsomal preparation was added to this system, together with GTP, phosphoenolpyruvate and pyruvate kinase, the presence of the drug resulted in an increased labelling of the transfer RNA and a decreased labelling of the microsomal protein compared with a control, drug-free system. The substitution of ribosomes, prepared by the method of Korner (1961), for microsomes gave a similar result.

When the pH 5·0 fraction was labelled with L-[^{14}C]leucine (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958) and then added to an appropriate microsomal preparation, chlortetracycline greatly reduced the transfer of the amino acid to the microsomal protein. This effect was increased by preincubating the microsomes with the drug, suggesting that its site of action was at the microsome. The inhibitory action of chlortetracycline was not released by raising the concentration of Mg^{2+} ions in the medium, indicating that its action is not merely one of removing these ions by chelation.

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The Incorporation of *p*-Fluorophenylalanine into Alkaline Phosphatase of *Escherichia coli*

By M. H. Richmond. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

When *E. coli* W organisms growing exponentially in a medium rich in inorganic phosphate are transferred with washing to a medium lacking inorganic phosphate, growth (as measured turbidimetrically) is markedly inhibited and the culture synthesizes alkaline phosphatase after a period of 10–15 min. in phosphate-free medium (Torriani, 1960). Addi-

tion of *p*-fluorophenylalanine (100 μg./ml.) at the onset of alkaline phosphatase synthesis has only a slightly inhibitory effect on the rate of enzyme synthesis. However, purification (Garen & Levinthal, 1960) and examination of the enzyme made under these conditions showed that *p*-fluorophenylalanine was present in the purified alkaline-phosphatase protein and that the phenylalanine content of the protein was correspondingly reduced. The degree of replacement of phenylalanine by the analogue was dependent on the concentration of analogue in the growth medium; with 100 μg. of *p*-fluorophenylalanine/ml. the replacement was 56%, at 30 μg./ml., 34%; at 10 μg./ml., 12%.

Examination of the physicochemical properties of the enzyme containing 56% phenylalanine replaced by the *p*-fluoro derivative showed that the sedimentation coefficients ($S_{20,w}$) of the protein before and after reduction and carboxymethylation were 5·7 and 3·1 respectively, which is (within experimental error) the same as found for normal purified alkaline phosphatase.

Normal alkaline phosphatase separates into at least three enzymically active components on starch-gel electrophoresis at pH 8·0. Analogue containing enzyme was found to behave similarly and to show identical mobilities with the normal enzyme. The specific enzyme activity of the normal and analogue-containing enzyme was the same within experimental error.

A replacement of up to 56% of the phenylalanine of alkaline phosphatase by the *p*-fluoro derivative has, therefore, no detectable effect on the physicochemical or enzymic properties of the enzyme. This is in sharp contrast to the case of exo-penicillinase of *B. cereus*, where a 70% replacement resulted in a fall of about 50% in specific enzyme activity (Richmond, 1960*a*, *b*).

I would like to thank Dr P. A. Charlwood for carrying out the analyses with the ultracentrifuge.

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Immunochemical Evidence of Increased Liver Tryptophan Pyrrolase Amounts Following Induction *in vivo*

By Olga Greengard and P. Feigelson. (*Columbia University College of Physicians and Surgeons, New York City, U.S.A.*)

The tryptophan-induced elevation of rat liver tryptophan pyrrolase activity *in vivo* (Knox, 1951) is associated with two distinct phenomena (Greengard & Feigelson, 1961): (*a*) an initial threefold rise in activity due to saturation of the endogenous apo-tryptophan pyrrolase with its haematin cofactor, and (*b*) a further two- to fourfold rise which cannot be attributed to conversion of apo- to holotryptophan pyrrolase. The aim of the present work was to determine whether (*b*) is due to another activation process operating in addition to (*a*), or whether it reflects an increase in the amount of the enzyme protein itself. Rabbit-antibody preparations, obtained against purified tryptophan pyrrolase (Feigelson & Greengard, 1961*a*) were added to liver cell sap of known tryptophan pyrrolase activity and the activity remaining after removal of the antibody–enzyme precipitate was estimated in the presence of optimum haematin levels. The tryptophan pyrrolase activity (μmoles kynurenine formed/g. liver/hr.) of normal liver cell sap was 4·7 and that of cell sap obtained from rats to which 1 g./kg. L-tryptophan had been administered 4 hr. previously was 14·5. This increase in activity was found to be accompanied by a proportionate increase in immunologically titratable tryptophan pyrrolase enzyme protein, in both intact and adrenalectomized rats. Tryptophan pyrrolase thus provides the first example of a mammalian enzyme in which increased amounts of enzyme protein, following substrate administration, have been directly measured. The present results are compatible with the suggestion (Feigelson & Greengard, 1961*b*) that the tryptophan-mediated saturation of apo-tryptophan pyrrolase with haem *in vivo* leads to a stimulated synthesis of the apo-tryptophan pyrrolase molecule.

The amount of liver tryptophan pyrrolase following the administration of 10 mg./kg. cortisone acetate has also been estimated by immunochemical titration. The results show that the threefold rise in tryptophan pyrrolase activity is associated with a comparable rise in the amount of tryptophan pyrrolase enzyme protein. Thus, an increase in the level of a specific enzyme protein is shown to accompany the previously demonstrated stimulation of amino acid incorporation into liver protein by cortisone (Korner, 1960; Feigelson, Gross & Feigelson, 1962).

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Inhibition of Deoxyribonucleic Acid Nucleotidyltransferase by a Liver Deoxyribonuclease

By MARGERY G. KELLOCK, R. M. S. SMELLIE and J. N. DAVIDSON. (*Department of Biochemistry, University of Glasgow*)

Cell-free extracts of many tissues including ascites-tumour cells or thymus can bring about the synthesis of DNA in the presence of primer polydeoxyribonucleotide and the deoxyribonucleoside triphosphates of adenine, guanine, cytosine and thymine under the influence of DNA nucleotidyltransferase (DNA-polymerase) (Gray *et al.* 1960). The reaction is followed by measuring incorporation of thymidine 5′-[^{32}P]monophosphate (TMP) from thymidine 5′-triphosphate (TTP) into DNA using the assay method of Bollum (1960) and is diminished in the presence of aqueous extract of normal rat liver tissue (Gray *et al.* 1960).

The inhibitory factor (or factors) in liver, which has been assayed against calf thymus transferase, has been purified and found to be a heat-labile protein. Its action could be due to (*a*) combination with, or destruction of, the enzyme, (*b*) hydrolysis of the deoxyribonucleoside triphosphates, or (*c*) interference with the DNA primer.

Preincubation of the enzyme with the inhibitory factor has little effect on the incorporation of TMP. Crude preparations of the inhibitor have triphosphatase activity which progressively diminishes with purification. Preincubation of the DNA primer with the inhibitor decreases the priming capacity of the DNA considerably and the deoxyribonuclease activity increases with purification.

The liver nuclease has an optimum pH of around 8·0 and requires Mn^{2+} or Mg^{2+}. It is inhibited by Ca^{2+} and also by increasing the ionic strength of the medium. DNA which has been heated to 100° for 10 min. is degraded approximately three times as rapidly as native DNA.

Liver nuclease differs from pancreatic deoxyribonuclease I and splenic deoxyribonuclease II. Pancreatic deoxyribonuclease attacks native DNA more rapidly than heated DNA and has different ionic requirements. When it is added to the DNA nucleotidyltransferase assay, at a concentration which gives a release of acid-soluble nucleotides comparable with that given by liver enzyme, no inhibition results. Splenic deoxyribonuclease II at a similar concentration in the transferase assay causes considerable inhibition, but differs from liver nuclease in pH optimum (4·6), in a lower Mg^{2+} requirement, and in requiring a much higher ionic strength.

Oligonucleotides produced by the action of liver nuclease on DNA inhibit in the presence of primer DNA, and resemble certain oligonucleotides described by Keir (1962).

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Incorporation of Thymidine by L Cells Infected with Herpes Virus

By A. A. NEWTON and S. MCWILLIAM (introduced by B. A. NEWTON). (*Biochemical Laboratory, University of Cambridge*)

It has been shown previously that infection of cells in tissue culture by herpes virus results in an increase in the DNA content of the cells (Newton & Stoker, 1958). The time of synthesis of this DNA in relation to the course of infection has been studied by following the uptake of ^{3}H-labelled thymidine by suspensions of infected mouse cells (strain L). The incorporation of this precursor into DNA is inhibited for 5–6 hr. after infection, but, preceding the appearance of new infectious virus by about 1 hr., there is a period of rapid incorporation of isotopically labelled material. This incorporation continues for 3–4 hr. and then ceases completely.

The decreased rate of thymidine incorporation which is observed immediately after infection of the cells by herpes virus could result from inhibition of any one of the steps by which extracellular thymidine is incorporated into nucleic acid. The formation of nucleotides from radioactive thymidine by the infected cells has therefore been studied in greater detail. The rate of entry of labelled thymidine into acid-soluble intracellular 'pool' material is unchanged in infected cells. The activity of the enzyme system by which thymidine is converted into thymidine triphosphate (TTP) has been assayed in broken cell preparations by estimating the proportion of thymidine phosphorylated by the method of Weissman, Smellie & Paul (1960). Under these conditions preparations of infected cells phosphorylated more than 70 % of the added thymidine in 1 hr.; control cells treated similarly were able to phosphorylate only 40–50 % of the thymidine in this time. Moreover, labelled thymidine diphosphate and TTP were found to accumulate in the whole cells incubated with radioactive thymidine in the 5 hr. following addition of herpes virus. At the same time the quantity of acid-soluble nucleotide material extractable from the cells increased. These results suggest that the decreased rate of thymidine incorporation into DNA observed during the lag phase of herpes virus growth is not due to inhibition of the conversion of thymidine into TTP, but rather to inhibition of the polymerization of nucleotides to form DNA.

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Activation of Hydrogen and Electron Transport Systems in a Virus-Infected Local Lesion Host

By G. L. Farkas and F. Solymosy (introduced by Committee). (*Research Institute for Plant Protection, Budapest, Hungary*)

Studies on virus-infected local lesion host plants indicated a marked increase in the activity of dehydrogenases in the tissues around the lesions as shown by the *in vivo* application of the triphenyl tetrazolium chloride technique (Farkas, Király & Solymosy, 1960). The aim of the present study was to elucidate the nature of the activated metabolism in this area.

Enzyme activities were measured in cell-free extracts from the tissues near the lesions and from the healthy areas of the same leaves (control) by standard spectrophotometric techniques (Colowick & Kaplan, 1955). *Nicotiana tabacum* var. White Burley plants infected with the para strain of tobacco mosaic virus were used.

Most of the enzymes studied were found to be activated in the tissues surrounding the lesions. The following average values of stimulation were obtained (control 100%): glucose 6-phosphate dehydrogenase 255%, 6-phosphogluconic dehydrogenase 305%, cytochrome oxidase 148%, $NADPH_2$-dependent quinone reductase 138%, $NADH_2$-dependent quinone reductase 169%, polyphenol oxidase 200%, '$NADPH_2$ oxidase system' (oxidation of $NADPH_2$ by crude extracts without adding extra carriers) 188%. No change was experienced in the activity of phosphohexoisomerase, pentosephosphate isomerase and malic dehydrogenase.

The above results indicate that mainly the hexose monophosphate-shunt dehydrogenases are activated and also, to a lesser extent, systems capable of transferring electrons from reduced dinucleotide coenzymes via polyphenols and polyphenoloxidase or via unknown carriers ('$NADPH_2$ oxidase') to the molecular oxygen of air.

Pentoses accumulate in the tissues studied and ribose 5-phosphate added to the extracts is but slowly metabolized. This suggests that the later stages of hexose monophosphate shunt cannot cope with the strongly activated first steps of the pathway.

The higher activity of a number of enzymes in the tissues around the lesions is most probably due to enzyme protein synthesis. This idea is supported by preliminary results obtained with ^{32}P indicating a higher turnover of ribonucleic acid in the affected area. As to the biological role of the observations described it might be assumed that the localized acquired resistance of plant tissues to viruses, i.e. the phenomenon that the tissues around the local lesions are as a rule resistant to reinfection (Yarwood, 1960; Ross, 1961) is to be explained by a competition between viral and enzyme protein synthesis.

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Studies on Encephalomyocarditis-Virus Protein

By Mary G. Hamilton, I. M. Kerr and T. S. Work. (*National Institute for Medical Research, London, N.W. 7*)

Encephalomyocarditis virus (EMC) is a small neurotropic murine enterovirus which can be grown conveniently *in vitro* in Krebs II mouse ascites-tumour cells. It has been purified and crystallized and contains 30% RNA and 70% protein. Although electron micrographs indicated that the virus had a subunit structure, it was not possible to determine from them the number or arrangement of the subunits (Faulkner, Martin, Sved, Valentine & Work, 1961).

In the present studies we have used immunological techniques employing a rabbit antiserum to crystalline EMC both to detect antigens in complex mixtures and to estimate their diffusion coefficients (Allison & Humphrey, 1960). Sedimentation coefficients were measured by zone centrifugation (P. A. Charlwood, unpublished experiments) and approximate molecular weights were calculated. In the case of material from crystalline virus measurements were also made in the analytical ultracentrifuge.

When crystalline virus of particle weight 10 million is dialysed against 0·03 M-ethanolamine, 0·15 M-NaCl at pH 10·5 (cf. Newmark & Myers, 1957), an antigen of molecular weight about 60 000 is obtained. On the other hand, the major EMC-specific antigen distinct from whole virus that can be detected in infected cell material has a molecular weight about 350 000. Treatment of this antigen with ethanolamine at high pH also gives rise to an antigen of molecular weight approximately 60 000. These results suggest that the antigens obtained from crystalline virus and from infected cell material are related and probably are structural subunits of the virus protein, the larger being an aggregate of 5 or 6 of the smaller units.

Experiments on the time and site of synthesis of the large antigen in the infected cell show that it is first detectable in the cell sap from 30 to 60 min. after the synthesis of viral RNA is initiated, i.e. at a time consistent with its being the protein which finally appears in the completed virus particle (cf. Martin & Work, 1962). However, we cannot at present prove that this material is a precursor rather than a by-product in the synthesis of virus.

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Tryptic Peptide Maps of Mutant *Salmonella* Flagellins

By M. W. McDonough. (*Guinness–Lister Research Unit, Lister Institute of Preventive Medicine, London, S.W.* 1)

Seven serologically distinct mutant forms of the phase 1 flagellar antigen *i* of *Salmonella typhimurium*, strain LT2 were found by Joys (1961) amongst eight spontaneous mutants obtained by selection in semi-solid medium containing a just-immobilizing concentration of anti-*i* serum. The wild-type and mutant forms of flagellar protein were prepared from monophasic *Salmonella* derivatives given the wild-type or mutant H1^{i} gene by transduction. Contamination of the phase 1 flagellin with phase 2 flagellin (antigen *1*, *2*) was thus avoided.

Bacteria grown on nutrient agar at 37° for 40 hr. were harvested, deflagellated by blendor treatment and bodies removed by centrifugation. The flagella were purified by alternate fast (105 000*g*) and slow (20 000*g*) centrifugation and dissociated at pH 2 (Weibull, 1948). Insoluble material was removed and the flagellin precipitated at 70% ammonium sulphate saturation, redissolved and dialysed at 4°.

The flagellins were denatured at 100° and digested with trypsin (enzyme to protein ratio 1:50) at pH 8·5 and 37° for 2 hr. Peptide maps were made by electrophoresis at pH 6·5, 3 kv for 1 hr., then chromatography in butanol–acetic acid–water (4:1:5, by vol.) for 30 hr. Peptides were detected with 0·2% (w/v) ninhydrin in acetone.

The maps revealed some thirty spots, sixteen basic, five acidic and nine neutral. Four of the mutant maps were indistinguishable from the wild-type map. In three of the mutants a slow-moving, basic spot of the wild-type was absent and a new basic spot was present. In mutant *i*M7 it was more basic, but chromatographically slower, while in *i*M10 and *i*M11 the new spots were alike with the same charge as the wild-type peptide but moving faster on chromatography.

Preliminary experiments with peptic digests have shown on electrophoresis the absence in *i*M9 flagellin of an acidic, tyrosine-containing band present in the wild-type and the other mutant proteins.

Thus in at least four of the seven mutants the change in antigenic character is accompanied by a chemical change, presumably in amino acid sequence. The serologically unrelated phase 2 flagellin (antigen *1*, *2*) of *Salm. typhimurium* gave a tryptic digest map in which only sixteen out of thirty spots corresponded in position to *i* peptides.

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Comparison of Creatine Phosphotransferase from Rabbit and Brown-Hare Muscle

By N. Dance and D. C. Watts.* (*Department of Biochemistry, University College London, Gower Street, W.C.* 1)

Creatine phosphotransferase was purified from rabbit and brown-hare muscle by parallel procedures to the precrystallization stage (Kuby, Noda & Lardy, 1954). The specific activities of the two preparations were found to be 61 and 60 Kuby units respectively. Starch-gel electrophoresis at a number of different pH values showed that both proteins migrated at the same rate and appeared to be at least 90% pure.

Comparisons of a number of the catalytic and chemical properties of the two enzymes were carried out. The Michaelis constants for creatine and ATP were not significantly different. The rate of inactivation with iodoacetate and iodoacetamide was the same for both enzymes and the substrate equilibrium mixture protected them to the same extent. In each case, one reactive sulphydryl group per particle weight of 40 000 was found, and the rate of alkylation of this was independent of pH between 6·0 and 9·7. Variations of ionic strength affected the rate of reaction of both enzymes with iodoacetate to the same extent. Thus, the two native enzymes cannot be distinguished by any of these criteria.

Both enzyme preparations were then denatured and hydrolysed with trypsin in a pH-stat at pH 9·5 and 37°. The peptides obtained were compared directly by high-voltage paper electrophoresis in a pyridine–acetic acid buffer at pH 6·4 and were also

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separated by electrophoresis followed by chromatography using a pyridine–isoamyl alcohol–water mixture (35:35:27, by vol.). Direct comparison of the two peptide patterns showed that most of the peptides appeared to be electrophoretically identical. However, two distinct peptide differences were found. Thus, while the kinetic evidence would suggest that the configuration of the protein in the vicinity of the active sites is the same in the two enzymes, the molecules are not completely identical.

The maximum number of peptides which could be detected by the two-dimensional analyses was 53 for each enzyme. The amino acid analysis of rabbit creatine phosphotransferase (Noltmann, Mahowald & Kuby, 1962) showed that there are 65 lysine and 34 arginine residues per molecule of 81 000 molecular weight so that tryptic hydrolysis would be expected to produce about 100 peptides. The appearance of approximately half this number of peptides suggests that rabbit muscle creatine phosphotransferase is composed of two similar subunits.

One of us (N.D.) wishes to thank the Department of Technical Cooperation for financial assistance.

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Absence of the Liver Enzyme, UDPG–Glycogen 1,4-Transglucosylase, as a Cause of Infantile Hypoglycaemia

By G. M. Lewis, K. M. Stewart and J. Spencer-Peet (introduced by G. H. Lathe). (*Departments of Chemical Pathology and Paediatrics, University of Leeds*)

Identical twins who have profound hypoglycaemia (less than 10 mg. of glucose/100 ml. of blood) about 12 hr. after a meal have been studied. One is mentally defective, the other below average development, and both have had convulsions after an overnight fast.

Plasma insulin was normal during fasting. Glucose tolerance tests indicated a reduced capacity to assimilate glucose, and a rise in blood glucose following glucagon administration in the fed, but not the fasted, state suggested a reduced capacity to store glycogen.

The possibility that this was due to abnormality, or absence, of an enzyme was considered. A defect in hexokinase was eliminated by administering fructose and galactose, which would bypass this enzyme. These sugars did not prevent subsequent hypoglycaemia.

A liver biopsy of one of the twins in the fed state confirmed the glycogen deficiency (0·5% glycogen, 10% lipid). There was complete absence of glycogen synthetase activity, and normal amounts of phosphorylase, UDPG pyrophosphorylase and glucose 6-phosphatase.

The infants did not respond to hypoglycaemia by increasing their adrenaline excretion. It is suggested that hydroxylations in the adrenal gland may be blocked by an inability to maintain $NADPH_2$ in the absence of carbohydrate stores.

The father and three siblings have abnormalities of carbohydrate metabolism. The family incidence, and the occurrence of the same condition in twins, suggest that it is an inborn error of metabolism.

Reduced glucose tolerance and an absence of hypoglycaemia during 5 hr. after a meal are not usual findings in idiopathic hypoglycaemia of infancy, but cases reported by Broberger & Zetterström (1961) appear to be similar. The term 'glycogen storage deficiency' is suggested.

Giving the affected infants a meal at midnight prevents them from becoming hypoglycaemic before breakfast.

Broberger, O. & Zetterström, R. (1961). *J. Pediat.* **59**, 215.

Peptiduria in an Unusual Bone Disorder

By J. W. T. Seakins. (*Hospital for Sick Children, Great Ormond Street, London, W.C.* 1)

During the routine screening by paper chromatography of mentally retarded children for abnormalities in amino acid metabolism, a child was discovered (then aged 2 years) whose urinary amino acid pattern showed the presence of a substance (Y) which gave a yellow, changing to mauve reaction with ninhydrin and a blue isatin reaction, and which had the same chromatographic parameters as proline. Present also on the chromatogram were a number of unidentified ninhydrin positive substances which disappeared on hydrolysis. In addition, the urine contained a 'protein' not retarded on Sephadex G-50 which on hydrolysis gave the same amino acid pattern as hydrolysed collagen. The excretion of glycine was raised, but the serum amino acid pattern was normal. The alkaline phosphatase during the last 2 years of life varied from 110 to 600 King–Armstrong units per 100 ml. of serum (normal 10–22). There has been a continuous thickening of the whole of the skeleton with the exception of the hands and feet which do not appear to be involved.

Displacement chromatography (Westall, 1952) of the urine firstly on Zeo-Karb 225 and secondly of

appropriate fractions on De-Acidite FF gave the substance (Y) substantially free from other amino acids, urochrome, creatine and creatinine. Further purification was effected by thick-paper chromatography. Hydrolysis of the substance gave glycine, proline and hydroxyproline. Edman degradation gave as *N*-terminal amino acids glycine and proline with the former predominating. Proline and, in smaller amounts, hydroxyproline only were present in the aqueous layer. Authentic glycylproline has the same R_F values as the predominant component in six different solvent systems, is not separated from it by high voltage electrophoresis and gives the same ninhydrin colour reaction. The two components can be separated from each other by elution chromatography, and again the predominant component emerges with glycylproline. Preliminary experiments suggest that the minor component is prolylhydroxyproline. Approximately 300 mg. of glycylproline, 100 mg. of the other peptide and 'protein' equivalent to 300 mg. of collagen were excreted daily.

The presence of large amounts of bound urinary hydroxyproline indicates considerable collagen turnover (Prockop & Sjoerdsma, 1961), which is consistent with the biochemical and clinical picture. The relationship between the disease process, the urinary peptides and 'protein' was discussed.

The author thanks Professor Alan Moncrieff for permission to study his patient.

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Chelates of Dihydrolipoic Acid in Relation to the Heavy-Metal Inhibition of α-Oxoglutarate Dehydrogenase

By M. Webb. (*Strangeways Research Laboratory, Cambridge*)

The intermediary formation of the dithiol form of the lipoic acid coenzyme during the turnover of the keto acid dehydrogenases renders these enzymes particularly susceptible to inhibition by ions of heavy metals (Sanadi, Langley & White, 1959; Veeger & Massey, 1960). Inhibition of α-oxoglutarate dehydrogenase by certain cations is reversed readily by 2,3-dimercaptopropan-1-ol (BAL), as previously observed with the Cd^{2+}-inhibited enzyme by Sanadi *et al.* (1959). Under the appropriate conditions, however, inhibition of α-oxoglutarate dehydrogenase by Co^{2+} and Cu^{2+} is not reversed by BAL. These differences are explicable in terms of the properties of the chelates of dihydrolipoic acid with various cations.

Anaerobically, dihydrolipoic acid and Co^{2+} combine in a 2:1 ratio with the liberation of one hydrogen ion/mole ligand, both thiol groups of the latter being involved in the formation of the chelate. In air this chelate undergoes immediate oxidation with an increase in molar extinction coefficient ($\epsilon_{max.}$) from $5{\cdot}12 \times 10^3$ (at 480 mμ) to $1{\cdot}45 \times 10^4$ cm.²/mmole (at 510 mμ). The addition of stoicheiometric amounts of Co^{2+} to dihydrolipoic acid at pH 7–8 is followed by a rapid uptake of 1·5 atoms of oxygen/mole of chelate, and the development of the colour characteristic of the oxidized complex. After these changes neither dihydrolipoic acid, nor biologically-active derivatives of lipoic acid can be recovered.

The rate of oxidation of dihydrolipoic acid at pH 8 is also increased by Cu^{2+} and Ni^{2+}. The initial rates of oxidation of chelates of these cations, however, are considerably less than that of the Co^{2+} derivative. Moreover, oxygen uptake by dihydrolipoic acid in the presence of varying amounts of Cu^{2+} is essentially independent of the cation content, whilst oxidation of the Ni^{2+} chelate is unaccompanied by changes in extinction. The mechanism of oxidation of these chelates, therefore, differs from that of the Co^{2+} derivative. Nevertheless, the Cu^{2+}-dihydrolipoic acid chelate is extremely stable, and cannot be dissociated to yield the ligand in a biologically-active form.

In contrast, chelates of dihydrolipoic acid with Zn^{2+} and Cd^{2+} are stable in air, and in the presence of stoicheiometric concentrations of either of these cations, the oxidation of the dithiol is inhibited completely. These chelates dissociate in acid solution with the quantitative liberation of coenzyme activity.

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The Degradation of Human Aortic Wall with Elastomucoproteinase

By Ilona Banga. (*I. Department of Pathological Anatomy and Experimental Cancer Research, Medical University, Budapest*, VIII, *Üllói ut* 26, *Hungary*)

In the elastolysis of elastic fibres mucolytic as well as proteolytic processes are taking part, as suggested first by Hall, Reed & Tunbridge (1952). Hall (1953) and Hall & Gardiner (1955) showed the liberation of organically bound acid and they suggested that sulphate ion is bound to mucopolysaccharide which is liberated during the lysis of elastic fibres. The liberation of polysaccharides is catalysed by elastomucoproteinase (EMP-ase) (Banga & Baló, 1956), but the mode of action is

unknown. Hall (1957) and Loeven (1960) showed a synergistic effect between elastase and elastomucoproteinase.

The properties and the mobility in the electric field of elastase and EMP-ase are very similar, which makes the separation of the two enzymes very difficult. We succeeded by preparative starch-gel electrophoresis in obtaining an enzyme preparation which had practically no effect on isolated elastin, but showed measurable proteolytic activity with an acetone-dried powder of human aorta. The aortic-elastolysate obtained with the above enzyme contained besides protein large quantities of acid and neutral mucopolysaccharides (20%) and also lipid (18–25%). The aortic elastolysates obtained by elastase contained much less mucopolysaccharides (3–4%) and their lipid content was also very low (5–6%). The EMP-ase therefore can be considered as a mucolipoproteinase which dissolves a mucolipoprotein from the human arterial wall.

In histological section, EMP-ase like elastase dissolves the elastic fibres in spite of the fact that the isolated elastin cannot be dissolved by EMP-ase. This phenomenon can be explained, if the elastic fibres are enveloped by a sheath or membrane consisting of the mucolipoprotein which is dissolved by EMP-ase. After dissolution of the sheath the elastic fibres disintegrate and lose their structure and staining properties.

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Observations on the Respiratory Quotient of Man after Eating Large Quantities of Carbohydrate

By R. Passmore and Y. E. Swindells. (*Department of Physiology, University of Edinburgh*)

That carbohydrate is fattening is well known. The conversion of carbohydrate into fat is associated with high R.Q.'s, as was shown in the classical experiments of Pembrey (1901–02). He found R.Q.'s ranging from 1·04 to 1·39 (mean 1·21) in marmots preparing for hibernation. These observations might lead one to think that it would be easy to demonstrate the conversion of carbohydrate into fat in man by finding high R.Q.'s. This has not proved to be the case.

We have fed seventeen subjects a diet of bread, jam and fruit, which is practically fat-free, in amounts far in excess of their calorie needs and followed the respiratory exchanges at intervals for periods from 14 to 36 hr. In all 218 measurements of the R.Q. were made. Of these, 21 reached 1·0, but only 3 were above 1·04. In 9 subjects the metabolic mixture was calculated for a 24 hr. period of overfeeding. The mean value for the carbohydrate metabolized was 450 g., against a mean intake of 820 g. In only one subject was the net utilization of fat completely suppressed. It was calculated that he had laid down a net amount of 18 g. of fat, but only after eating 1·1 kg. of carbohydrate.

These results could be explained if there were a delay in the conversion of carbohydrate into fat and the excess carbohydrate was first stored, perhaps as glycogen. Such excess glycogen would amount to up to 500 g., a quantity greater than is usually found in the whole human body (Soskin & Levine, 1952). Most of it would have to be stored in muscle, in which the concentration would have to rise by as much as 1·5 g./100 g.

A similar failure to find a high R.Q. after feeding carbohydrates was reported by Furnass (1960). He suggested that 'unexpectedly low respiratory quotients may represent the combination of some absorbed oxygen with hydrogen atoms in depot fat thereby resulting in desaturation of the fat'.

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Metabolism of [4-^{14}C]Progesterone by Adrenocortical Tissue from Patients with Cushing's Syndrome

By Pamela J. Ward* and J. K. Grant. (*University Department of Steroid Biochemistry, Royal Infirmary, Glasgow*)

Adrenal glands were removed surgically from two patients with Cushing's syndrome: (*a*) a 46-year-old man with bilateral adrenal hyperplasia (weight of gland studied 7·6 g.), and (*b*) a 15-year-old girl with bilateral adrenal hyperplasia (weight of gland studied 5·7 g.). An adrenal tumour, weighing 139 g., was removed from a third patient, a 15 year old girl with Cushing's syndrome accompanied by severe virilism.

Slices of cortical tissue (predominantly 'compact' cells in patient (*b*)) from the hyperplastic glands and

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slices from the tumour (predominantly 'clear' cells) were incubated in an atmosphere of 95% O_2–5% CO_2 at 37° for 3 hr. in Krebs–Ringer bicarbonate solution containing glucose, corticotrophin (Organon A_1—200 mu./100 mg. of tissue) and [4-^{14}C]progesterone (3 μc, 50 μg./100 mg. of tissue). 'Compact' and 'clear' cells are those defined by Symington (1960).

Radioactive substances were extracted from the incubation mixtures, separated and provisionally identified by paper chromatography in several different solvent systems. After elution of these substances, known non-radioactive steroids were added and further identification was achieved by extensive paper chromatography and specific activity measurements before and after the preparation of derivatives.

The main metabolic products were 17α-hydroxyprogesterone, 16α-hydroxyprogesterone, corticosterone and 11β-hydroxyandrost-4-ene-3,17-dione. 11-Deoxycorticosterone, androst-4-ene-3,17-dione and testosterone were formed in lesser amounts.

The formation of 16α-hydroxyprogesterone and testosterone from progesterone by adult human adrenal cortex has not hitherto been described. Villee, Engel, Loring & Villee (1961) have reported the formation of 16α-hydroxyprogesterone as well as 17α-hydroxyprogesterone and 11-deoxycorticosterone from progesterone by human foetal adrenal slices. Anliker, Rohr & Marti (1956) detected testosterone in extracts of a virilizing adrenal tumour.

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The Selection of a Suitable Control for Studies on Liver Regeneration

By P. B. Gahan. (*Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C.* 2)

In seeking a control for normal liver biochemistry, Dale (1962) selected laparotomy under ether anaesthesia followed by rapid freezing of the tissue. It can be shown, histochemically, that these procedures cause profound changes in normal rat liver (Bitensky & Gahan, 1962). This raises the problem of what to use as the control tissue for biochemical studies on regeneration of rat liver.

A histochemical study of the right liver lobe from animals treated by ether anaesthesia followed by laparotomy and handling of the left and ventral liver lobes (I) showed some of the changes associated with regeneration. Liver cell disturbances also occurred with ether anaesthesia (II) or ether anaesthesia followed by laparotomy (III), but to a markedly lesser degree than in (I). Particularly noticeable was the appearance of many neutral fat droplets and an increase in the availability to staining of cytoplasmic phospholipids, which occurred most prominently 5 hr. after treatment (I) in Glaxo Piebald Strain rats and at 24 hr. in August rats. Biochemical estimations showed an increase in liver-lipid content of August rats at 24 hr., but no significant changes in the lipid levels of the Glaxo rats. These changes suggest an alteration in the state of the cytoplasmic lipids in those livers of animals which have been subjected to these treatments. Because such changes were not manifest until some time after operation they would not be detectable in any lobes which had been removed and which are often used as control tissue, and hence this tissue does not seem to be an adequate control to regeneration. It seems also that laparotomy alone is insufficient and that handling of the liver at least is required, so confirming the use of this latter control by Weber & Cantero (1955).

These findings will be discussed with respect to the evaluation of some of the changes associated with liver regeneration which have now been shown, in part, to result from the effect of the operation.

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The Development of Hepatic Hexokinases after Birth

By D. G. Walker. (*Department of Biochemistry, University of Birmingham, Birmingham* 15)

The first enzymic stage in the utilization of monosaccharides by the liver is phosphorylation by ATP in the presence of a hexokinase. Both specific and non-specific hexokinases have been reported as being present in animal tissues (Crane & Sols, 1955) and their varying properties noted.

A specific liver fructokinase was described by Hers (1952), who later reported (Hers, 1957) the absence of this enzyme from the liver of the foetal sheep, a species having fructose as the principle foetal blood sugar. This absence of foetal hepatic fructokinase is now shown to be a more general phenomenon. Fructokinase activity has been estimated by measuring fructose disappearance in an incubation medium including ATP, Mg^{2+} ions and

also creatine phosphate and creatine phosphokinase to reconvert inhibitory ADP back into ATP (Parks, Ben-Gershom & Lardy, 1957). Hepatic fructokinase develops immediately after birth and reaches adult levels after about 7 days in the guinea-pig and rabbit, these being non-fructogenic species. The absence of activity in the foetal liver is not due to an inhibitor. Placental tissue shows no specific fructokinase activity.

Glucose phosphorylating activity has been estimated in the supernatant fraction of liver homogenates by measuring glucose 6-phosphate formation at pH 7·5 directly by coupling with glucose 6-phosphate dehydrogenase and $NADP^+$ (DiPietro & Weinhouse, 1960). Adult guinea-pig liver can phosphorylate 0·5–1·0 μ-mole of glucose/min./g. of tissue. Foetal and new-born livers show lower activities, whereas placental tissue shows similar activity to adult liver.

Using the same assay procedure attempts have been made to study the nature of the enzymes responsible for this activity. The assay method has limitations for kinetic and specificity studies on crude tissue preparations. Attempts to purify the enzymes have shown them to be very labile. Using dialysed preparations, distinct differences between foetal and adult systems are observed. Foetal liver has a K_m for glucose of about 3×10^{-5} M with a K_i for 2-deoxyglucose of approx. 6×10^{-4} M. Galactose also inhibits this enzyme. The placental enzyme is similar. The adult liver contains two enzymes, one having a K_m similar to that in the foetal liver and the other having a K_m of 3 to 4×10^{-3} M. The latter enzyme, probably a specific glucokinase, thus also develops in the guinea-pig liver as another example of neonatal hepatic maturation.

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Metabolism of Compounds Related to Mustard Gas

By J. J. Roberts and G. P. Warwick. (*Chester Beatty Research Institute, Fulham Road, London, S.W.* 3)

Ethylmethanesulphonate ($CH_3 \cdot SO_2 \cdot O \cdot CH_2 \cdot CH_3$) and Myleran ($CH_3 \cdot SO_2 \cdot O \cdot [CH_2]_4 \cdot O \cdot SO_2 \cdot CH_3$) react extensively with cysteine-containing compounds *in vivo* giving *S*-ethylcysteine derivatives and 3-hydroxytetrahydrothiophen 1,1-dioxide respectively, as urinary metabolites (Roberts & Warwick, 1958, 1961*a*, *b*).

This transformation involves the intermediate formation of a cyclic sulphonium ion and its decomposition to tetrahydrothiophen, involving removal of the sulphur atom from the cysteinyl moiety (dethiolation).

The affinity of these alkylating agents for the thiol group indicated that the monofunctional and bifunctional sulphur and nitrogen mustards might behave similarly.

While Myleran reacted with cysteine and cysteine-containing compounds *in vitro* to form a cyclic sulphonium ion which could react with various nucleophilic species, the bifunctional mustards, e.g. bischloroethyl sulphide (H) and *NN*-bis-(2-chloroethyl)aniline did not form stable sulphonium ion intermediates but yielded biscysteinyl compounds, although labile sulphonium ions may be involved in the reaction.

For example:

$$HS \cdot CH_2(NH_2) \cdot CO_2H + Ph \cdot N(CH_2 \cdot CH_2 \cdot Cl)_2 \rightarrow Ph \cdot N[CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H]_2$$

Similar reactions occur *in vivo* and with bischloroethyl sulphide (mustard gas) one of the major urinary constituents following intraperitoneal injection and hydrolysis of the urine is

$$O_2S[CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H]_2$$

which would not have been anticipated had the mustard behaved similarly to Myleran.

Difficulty was encountered in studying the metabolism of the monofunctional mustard *N*-2-chloroethyl-*N*-ethylaniline

$$CH_3 \cdot CH_2 \cdot NPh \cdot CH_2 \cdot CH_2 \cdot Cl.$$

By analogy with ethyl methanesulphonate the formation of a simple mercapturic acid was anticipated. Following intraperitoneal injection of *N*-(2-chloro-[1,2-$^{14}C_2$]ethyl)-*N*-ethylaniline 80% of the radioactivity was recovered in the urine and faeces. Many radioactive areas were present on radioautographs, one metabolite accounting for about 50% of the urinary radioactivity. However, spray reagents failed to produce evidence for the presence of a mercapturic acid or a sulphur-containing metabolite.

Examination of the urine from rats injected with the corresponding radioactive cysteine and glutathione derivatives failed to add additional evidence.

As an alternative approach the body sulphur pool of two rats was labelled by administration of L-[^{35}S]cystine, and the unlabelled mustard was

given to one animal (B), the other being a control (A). A third rat (C) was injected with the ^{14}C-half mustard. Radioautographs of chromatograms of the urines were compared and the additional radioactive areas present in the urine of B were identical to those present in the urine of C. Presumably the metabolites were derived from the product of initial reaction with the thiol group, probably of a cysteinyl moiety.

Chloroethyl hydroxyethyl sulphide was converted in the rat almost exclusively into metabolites of thiodiglycol.

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Sodium Selenite as a Catalyst for the Reduction of Methylene Blue by Sulphydryl Amino Acids

By M. G. STANTON (introduced by T. MOORE). (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Evidence that selenium may have a role in biological systems was advanced by Pinsent (1954) who reported that selenite, together with molybdate, was necessary for the formation of formic dehydrogenase in the *coli aerogenes* group of bacteria. More recently selenium has been found the active agent in Factor 3 of Schwarz (1951) (Schwarz & Foltz, 1957). Selenium proves active against some, but by no means all, of the effects of vitamin E deficiency. Thus, very small doses of selenium protected rats against dietary liver necrosis (Schwarz & Foltz, 1957) and chicks against exudative diathesis (Patterson, Milstrey & Stokstad, 1957). The biochemical mode of action of selenium is still unknown, but Gitler (1958) has described a model system by which he demonstrated that the reduction of methylene blue by cysteine is catalysed by sodium selenite.

Experiments were undertaken to confirm and extend the observations of Gitler. Reduction of methylene blue by cysteine was first investigated under the conditions reported by Gitler, at 70° and pH 7·4. Catalysis was certainly observed with selenite, but the results were very inconsistent. The inconsistencies were traced to the presence of atmospheric oxygen. Exclusion of oxygen by nitrogen so much accelerated the reaction that it was necessary to reduce the temperature to 40° and the pH to 6·0 in order to overcome practical difficulties. The progress of the reaction was followed in a photoelectric absorptiometer fitted with cells, specially designed to facilitate a speedy mixture of the reactants and also the exclusion of oxygen.

The catalysis of homocysteine and glutathione by sodium selenite was also investigated by the same method. Both these substances showed considerably greater catalysis by selenite than did cysteine. The concentration of catalyst was only taken up to 80 micromolar for practical reasons. At this concentration the catalysis of glutathione was showing signs of reaching a limit, but for cysteine and homocysteine the catalysis was still increasing.

Tellurium, which appears in the same group as selenium in the Periodic Table, was tested, in the form of potassium tellurite, in anticipation that a comparison of its action with that of selenium might prove helpful. Tellurite had some catalytic effect with both cysteine and homocysteine, but was almost inactive with glutathione. The inferiority of tellurite to selenite as a catalyst was in agreement with the finding of Patterson *et al.* (1957) and Schwarz *et al.* (1957) that it is also much inferior in biological potency.

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Studies on the Ceric–Arsenite Reaction for Detection of Organic Iodine

By S. B. BARKER. (*Department of Biochemistry, University of Edinburgh and Department of Pharmacology, University of Alabama Medical Center, Birmingham 3, Ala., U.S.A.*)

When a constant volume of 10 μl. of varying concentrations of iodine-containing solutions is applied to chromatographic filter paper, the Bowden–Maclagan–Wilkinson (1955) ceric arsenite reagent can yield semiquantitative results upon visual comparison of spots of decolorization. Using this technique, we have found that various iodothyronines give about 8% as much decolorization as equivalent amounts of iodide. This agrees with the relative amounts of I_2 obtained by Bowden *et al.* (1955) following ceric oxidation of iodothyronines and of iodide although 3,5-di-iodothyronine could be detected with high sensitivity on paper.

Any combination of 3-, 5-, 3′-, or 5′-positioning of the iodine on the thyronine molecule is effective. Alkyl substitution at 2′, 3′, or 5′ does not alter the reactivity of 3,5-di-iodothyronine (3,5-T_2), but reactivity is lost in several 4′-deoxy compounds. Changing the hydroxyl of 3,5-T_2 to the 2′ or 3′ position on the β-ring results in no loss of decolorization. Both 4′-methyl-4′-deoxy and 4′-methyl ether types of compounds are unreactive. Replacement of the 4′-hydroxyl by 4′-amino permits reactivity.

Similar differences are obtained in solution, but with the considerably more dilute reagents employed, decolorization with iodothyronines is more nearly equivalent to that with iodide.

If decolorization is considered as resulting from some form of iodine liberated by ceric ions from organic combination, it is suggested that an ionizable substitution must be present on the outer aromatic ring of 3,5-di-iodophenyl-4-phenoxyalanine for deiodination to occur. It is not known whether this β-ring must be lost as an obligatory preliminary to deiodination or whether free-radical formation at the ether oxygen should be postulated.

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Methaemoglobinaemia Induced by 4,4′-Diaminodiphenyl Sulphone

By M. Hjelm and C.-H. de Verdier (introduced by Committee). (*Department of Clinical Chemistry, University Hospital, Uppsala, Sweden*)

During treatment of leprosy and dermatitis herpetiformis with 4,4′-diaminodiphenyl sulphone (avlosulphone) methaemoglobinaemia and anaemia frequently occur as a complication (Imperial Chemical Industries, 1955; M. Skogh, unpublished results).

By tagging the red blood cells with radioactive chromium it was shown that the anaemia has a haemolytic nature. Incubation of the erythrocytes in three different media, one containing plasma before treatment, the other plasma before treatment plus added 4,4′-diaminodiphenyl sulphone, and the third plasma after 2 days treatment with the drug, revealed that a metabolite of the drug is responsible for the methaemoglobin formation. Some experiments performed to elucidate the mechanism of action of this metabolite will be described.

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DEMONSTRATION

A Method of Scanning Cellulose Acetate Membrane Electrophoresis Strips for Radioactivity and Light Absorption

By U. E. Loening. (*Department of Botany, University of Edinburgh*)

The radioactivity of the strips is determined by scintillation counting. For the counting of ^{14}C and ^{3}H the membranes are cleared in a scintillation fluid of suitable refractive index; for ^{32}P a scintillation plastic close to the membranes is used. The strip is transported past a slit by a ratchet operated drum, the whole device being fitted to the Ekco N 664 A scintillation head and electrically actuated. Strips 10 cm. by 1–1·5 cm. can be counted at 1·5–3 mm. intervals. An apparatus to hold larger strips is being designed. The Ekco head requires no modification except for fitting the electrical connexions.

Light absorption can be determined down to 240 mμ when the membranes are cleared in liquid paraffin or in Ondina Oil 17 (Scottish Oils and Shell Mex Ltd.). A replacement lid to the micro-cell housing of the Cambridge Unicam holds a drum which transports the cleared strips past the light path. The slit width and scanning intervals are adjusted to match those of the radioactivity determinations.

This apparatus was used to study the incorporation of ^{32}P into messenger and ribosomal RNA, as reported at this meeting.

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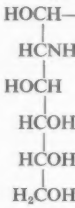
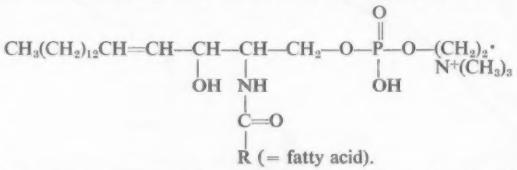


M.p. 185° c (dec.) (lit. 185·7° c (dec.)), $[\alpha]_D^{20} -32{\cdot}8 \pm 0{\cdot}4$ (c = 1 in H_2O). 500 μg. shows one spot on chromatogram. R_F = 0·2 (*n*-butanol : pyridine : H_2O (6:4:3)). Σ at 575 mμ Orcinol/Cu, 5890 (lit. 5900); at 580 mμ Resorcinol/Cu, 7950 (lit. 8000). 99 % pure by Periodate oxidation.

100 mg. at £8 500 mg. at £37 1 g. at £70. 10 g. at £60 g.

SPHINGOMYELIN, ex beef brain, highly purified



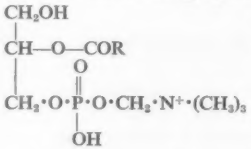
R (= fatty acid).

IR spectrum agrees with standard[1] and confirms absence of carbonyl ester linkages (e.g. Lecithin).
P = 3·9–4·1 % (theoretical 3·7–4·3 % depending upon R).

[1] Shapiro, D. and Flowers, H. M., *J. Amer. Chem. Soc.* **84**, 1047 (1962).

100 mg. at 62*s*. 1 g. at £25 10 g. at £20 g.

LYSOLECITHIN, enzymically by *Crotalus adamanteus* on chromatographically purified egg lecithin



Chromatogram on Silicic acid-coated paper reveals trace of Sphingomyelin.

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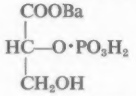
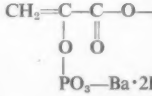
2-PHOSPHOGLYCERIC ACID, mono Barium salt
(D(+)-Glyceric acid-2-phosphate, Ba)



$[\alpha]_D$ + 24° (in N-HCl).

100 mg. at 16*s*. 1 g. at £6 10 g. at £5 g.

2-PHOSPHO*ENOL*PYRUVIC ACID, Silver, Barium salt dihydrate



Total P = 6·95 %.
Inorganic P = Nil.
Enzymically assay 94 %.

100 mg. at 30*s*. 1 g. at £12 10 g. at £10 g.

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α-Amylase
β-Amylase
Arginase
Bromelain
Catalase
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Chymotrypsins
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Coenzyme A
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Cytochrome C
DNase I & II
Diaphorase
DPN & DPNH
Ficin
FAD
β-Glucosidase
β-Glucuronidase
Hyaluronidase
Invertase
Lactase
Lactic dehydrogenase
Lipase
Lipoxidase
Lysozyme
Malic dehydrogenase
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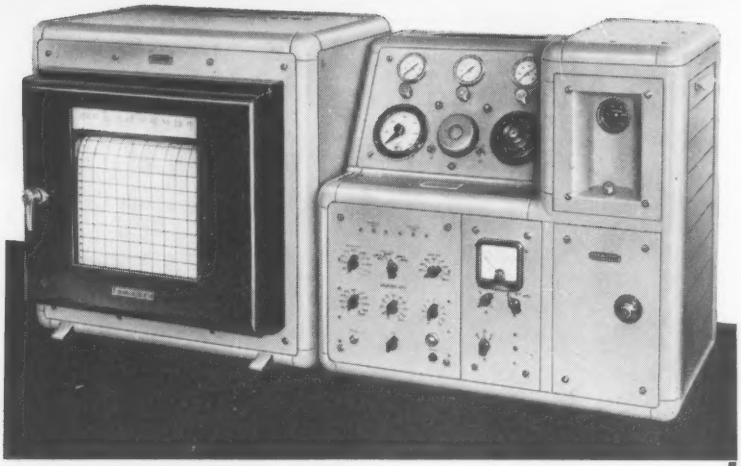

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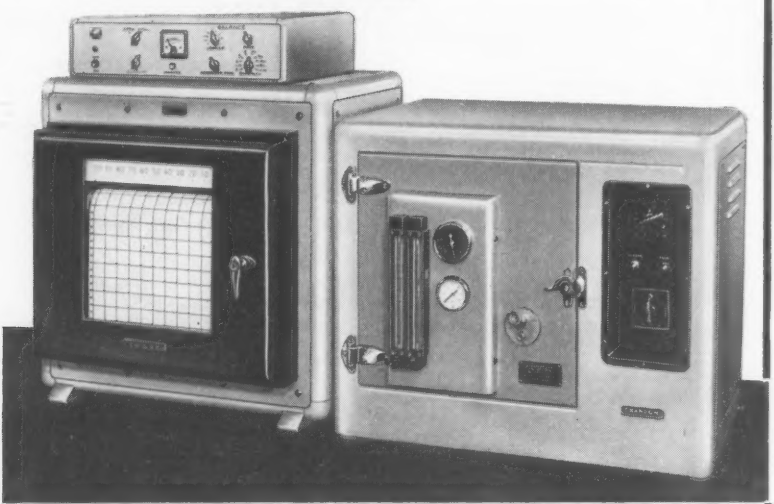

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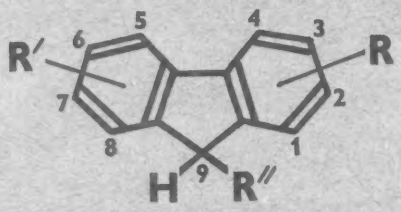

Have you condsiered trying the fluorene nucleus? Its known chemistry ranges widely from studies, sometimes considerably more complex and challenging than with single-ring compounds, of effects on substitution by various groups in the same or in the opposite ring (1); to optical isomerism involving the 9-position (2); to one of the few examples in the literature of *syn-anti* isomerism of >C=N—Ar (anils) (3); to modifications and metabolites of its powerful carcinogenic derivative, 2-acetamidofluorene (4) (this also has insecticidal properties (5) which obviously cannot be utilized); to two unusually acidic hydrocarbons based on or related to the fluorene nucleus (6); to its interesting, reactive, and dermatologically irritating derivative, 9-bromofluorene and related compounds (7); to the first example of an isomaleimide isolated from the ordinary type of closure to a normal maleimide (8).

There is the possibility of new benzofluoranthene derivatives (9) through closure of 9-*o*-chlorobenzylidene fluorene.

Dye chemistry, using the fluorene nucleus, is scattered through the literature all the way to India and Japan (10). Certain hydrofluorenes have been used in an approach to *c*-nor-*d*-homosteroids (11). And a new fungicide, 1,2,3,4-tetrahydrofluorenone, is described (12).

A number of analytical applications have been developed, for example (13).

Finally, in no way exhausting the subject, in breadth or depth, various natural products are known to contain the fluorene skeleton: gibberellic acid (14), condurangin and vincetoxin (15).

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